RED CELL ADHESION MOLECULES, FOETAL HAEMOGLOBIN AND ENDOTHELIAL FACTORS IN SICKLE CELL DISORDERS

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ABSTRACT

Sickle cell anaemia (SS) is a haemoglobinopathy involving production of sickle haemoglobin (HbS, β^6Glu→Val), which is able to polymerise leading to vaso-occlusion. Hydroxyurea (HU) treatment increases foetal haemoglobin (HbF) levels but decreases vaso-occlusion and red cell adhesion molecule (AM) expression, and therefore improves clinical symptoms. In this thesis, the contribution of AMs, HbF and endothelial factors to the severity of sickle cell disease has been studied.

Flow cytometry for measurement of HbF-containing red cells (F^+cells), AM-expressing red cells (AM^+cells) and reticulocytes (retics) was developed and validated using single-, double- and triple-colour staining procedures. The AMs examined were CD36, CD41 and CD49d.

F^+cells are increased in SS patients over normal control subjects. In the controls, the percentage of F^+mature red cells (%F^+MRCs) is equal to %F^+retics leading to an enrichment ratio (ER = %F^+MRCs / %F^+retics) close to 1.0, indicating no survival advantage of F^+cells. However in SS patients, the ER is about 1.5–2.5 indicating a survival advantage of F^+cells. AM^+cells in SS patients are also increased over the controls. In both the controls and SS patients, the AM^+cell depletion ratio (AMDR = %AM^+MRCs / %AM^+retics) is decreased to less than 0.05, indicating a rapid shedding of AMs from reticulocytes during maturation. This shedding is confirmed by a reduction of %AM^+cells to undetectable levels after 5 days of reticulocytes in culture. In HU-treated SS patients, F^+cell increment and AM^+cell reduction are found. The %AM^+retics for pre- and post-HU treatment is not different, suggesting that AM^+cell reduction is dependent on the reduction of reticulocytes. In the controls, proportion of F^+cells expressing AMs is higher than F^-cells, indicating that F^+cells are more primitive. In SS patients, these proportions are similar. However, in HU-treated SS patients, the proportion of F^+cells expressing AMs is lower than F^-cells,

confirming that F^+cells survive longer so that they have more time to shed more AMs. This finding is similar in both reticulocyte and MRC populations.

Vascular endothelial growth factor (VEGF) may be involved in vaso-occlusion. It is significantly increased in SS patients. VEGF is able to induce nitric oxide metabolite (NOx) release. However in this thesis, NOx levels were not increased in SS patients and no correlation was found between NOx and VEGF levels. No relationship was observed between VEGF and either erythropoietin or Hb levels, suggesting that VEGF increment may not be due to generalised anaemia. Increases in soluble endothelial selectin (sE-Selectin) and soluble vascular cell adhesion molecule 1 (sVCAM-1) found in SS patients indicate endothelial activation. Beta-thromboglobulin (BTG) and platelet factor 4 (PF4) were also increased in SS patients, due to local platelet activation *in vivo*. Platelets as well as endothelial cells contain VEGF, therefore the increased VEGF levels in SS could be a consequence of local ischaemia resulting from vaso-occlusion and local platelet and endothelial cell activation.

TO

my parents, grand parents, sisters, brothers

and

my wife

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LIST OF ABBREVIATIONS

AA	= Homozygote for HbA gene or normal person
ACS	= Acute chest syndromes
AM	= Adhesion molecules
AM^+cells	= Adhesion molecule expressing red cells
AM^-cells	= Red cells without adhesion molecule
AMDR	= Adhesion molecule depletion ratio
AM^+MRCs	= Adhesion molecule expressing mature red cells
AM^-MRCs	= mature red cells without adhesion molecule
AM^+reticulocytes	= Adhesion molecule expressing reticulocytes
AM^-reticulocytes	= Reticulocytes without adhesion molecule
AS	= Heterozygote for HbA and HbS or sickle cell trait
BMT	= Bone marrow transplantation
BSA	= Bovine serum albumin
BTG	= Beta-thromboglobulin
CD36	= Glycoprotein IV or IIIb or thrombospondin receptor
CD41	= Glycoprotein IIb or alpha-IIb integrin
CD49d	= Alpha-4 integrin of VLA-4
Cell	= Red blood cell unless stated otherwise
CMV	= Cytomegalovirus
CPDA	= Citrate phosphate dextrose and adenine
CRP	= Complement regulatory protein
CTAD	= Citrate theophylene adenine and dipyridamol
CVA	= Cerebro vascular accident
DNA	= Deoxyribonucleic acid
2,3-DPG	= 2,3-diphosphoglycerate
EDTA	= Ethylene diamine tetra-acetate salt (Na or K)
ELISA	= Enzyme linked immuno-sorbent assay

LIST OF ABBREVIATIONS

(Continued)

ER	= Enrichment ratio
Epo	= Erythropoietin
FBC	= Full (complete) blood count or CBC
F^+cells	= Foetal haemoglobin containing red cells
F^-cells	= Red cells without foetal haemoglobin
FCS	= Foetal calf serum
FITC	= Fluorescein isothyocyanate
F^+MRCs	= Foetal haemoglobin containing mature red cells
F^-MRCs	= Mature red cells without foetal haemoglobin
F^+reticulocytes	= Foetal haemoglobin containing reticulocytes
F^-reticulocytes	= Reticulocytes without foetal haemoglobin
FCP	= X-linked F^+cells production locus
Fe^{++}	= Ferrous ion
GAG	= Codon (guanine-adenine-guanine) for glutamate
g/dl	= Gram per decilitre (concentration unit)
G6PD	= Glucose-6-phosphate dehydrogenase
GTG	= Codon (guanine-thymidine-guanine) for valine
Hb	= Haemoglobin
HbA	= Adult haemoglobin
HbA_2	= Adult haemoglobin two
HbC	= Haemoglobin C
HbS	= Sickle haemoglobin
HPFH	= Hereditary persistent foetal haemoglobin
HPLC	= High pressure liquid chromatography
HSs	= Hypersensitive sites
HU	= Hydroxyurea
KDa	= Kilo Dalton (molecular weight unit)

LIST OF ABBREVIATIONS

(Continued)

LCR	= Local control region
MHC	= Major histocompatibility
mm Hg	= millimetre of mercury (air pressure unit)
MoAb	= Monoclonal antibody
NO	= Nitric oxide
NOx	= Nitric oxide metabolites
PBS	= Phosphate buffered saline
PF4	= Platelet factor four
P/S	= Penicillin and streptomycin
PSR	= Proliferative sickle retinopathy
RBC	= Red blood cell
R^+cells	= RNA containing red cells or reticulocytes
R^-cells	= Red cells without RNA or mature red cells
Retics	= Reticulocytes
RFLP	= Restriction fragment length polymorphism
RNA	= Ribonucleic acid
RPE	= Rhodamine phycoerythrin
SC	= Heterozygote for HbS and HbC
sE-Selectin	= Soluble endothelium selectin
SS	= Homozygote for HbS gene or sickle cell patient
sVCAM-1	= Soluble vascular cell adhesion molecule one
TC	= Tri-Color® or Phycoerythrin-Cyanin 5 or P-Cy5
TO	= Thiazole orange
VEC	= Vascular endothelial cell
VEGF	= Vascular endothelium growth factor
VLA-4	= Very late antigen four
VNR	= Vitronectin receptor
vWF	= von Willebrand factor

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CHAPTER 1

GENERAL INTRODUCTION

1.1. OBJECTIVES AND SCOPE OF THIS THESIS

The broad objective of this thesis is to characterise factors that may influence the severity of sickle cell disease. Two factors, which are known to influence the sickling process, are the foetal haemoglobin (HbF) content and the expression of adhesion molecules on sickle red cells. In general, a high HbF content slows the rate of sickle haemoglobin (HbS) polymerisation within sickle red cells under hypoxic conditions, thus leading to a lower proportion of sickle or crescent red cells in the circulation and a milder disease process. On the other hand, any factor, which can increase the adhesion of red cells to endothelium, will slow blood flow and increase the risk of sickling. The latter mechanism has been increasingly recognised as important in recent years. It is not clear from current knowledge to what extent the beneficial effects of HbF or the deleterious effects of red cell-endothelial adhesion interact to determine the course of sickling process. In this thesis, a method to study HbF content and adhesion molecule expression on the same red cells has been developed and at the same time examines the age of the red cells by staining for RNA content. In this approach it is possible in principle to look at the influence of HbF content and adhesion molecule expression on survival of sickle red cells beyond the reticulocyte stage. This has not previously been investigated.

A second approach to examining the influence of erythrocyte-endothelial interaction on sickle cell disease is to measure markers of endothelial activation, platelet activation and anaemia in various states of sickle cell disease e.g. in steady state, in crisis and on hydroxyurea (HU) treatment. In the final chapter, these markers in the plasma e.g. vascular endothelial growth factors (VEGF), nitric oxide metabolites (NOx), soluble vascular cell adhesion molecule 1 (sVCAM1), soluble endothelial selectin (sE-selectin), beta-thromboglobulin

(BTG), platelet factor 4 (PF4), haemoglobin (Hb) and erythropoietin (Epo) which are believed to be influenced by erythrocyte-endothelial interaction were measured and compared.

In this introductory chapter, a brief description of the pathophysiology of sickle cell disease is given. This is followed by a review on the influence of HbF to the sickling process. Finally the importance of erythrocyte-endothelial interaction to the sickling process is reviewed.

1.2. SICKLE CELL DISORDERS

Sickle cell disorders are inherited or genetic diseases resulting from a structural mutation of the β-chain of haemoglobin gene resulting in the production of sickle haemoglobin (HbS) instead of normal adult haemoglobin (HbA). HbS production is the result of a single point mutation at the 6^{th} codon (β^6). The normal GAG codon is substituted by GTG (Marotta *et al.*, 1977b; Mears *et al.*, 1981). This substitution results in the insertion of a non-polar valine residue instead of a highly polar glutamic acid (β^6Glu→Val) in the β-globin chain that is recognised as β^S. Thus HbS has more positive charge than normal HbA. Therefore it migrates further than HbA in gel electrophoresis (Slightom *et al.*, 1997). The substitution leads to its ability to polymerise in the deoxygenated state or hypoxia and its solubility is reduced (Noguchi *et al.*, 1981). Low oxygen tension (such as in post-capillary venules where O_2 tension is about 40 mmHg) can induce HbS polymerisation. Solubility of deoxy-HbS is only 1% of the oxy-form (Perutz & Mitchinson, 1950). HbS polymer (tactoid) builds up within the red cells after its conformational change in the deoxy state. The polymer formation can induce membrane damage, increase rigidity and change the red cell shapes from normal biconcave to crescent or sickle forms (Mohandas & Evans, 1989). The condition is autosomal recessive in clinical terms but co-dominant in productional terms. Thus although heterozygotes (AS) have both Hb A and HbS in each red cell (<50% HbS and >50% HbA), clinical complications are seldom

seen (Ogunye & Ejiogu, 1982). The pathophysiology and basic knowledge of sickle cell disease (SS) is reviewed as follows.

1.3. SICKLE HAEMOGLOBIN

In general, haemoglobin is composed of two pairs of polypeptide chains called globin chains, and each chain is folded around a haem molecule. The different globin chains are similar in their molecular structure and amino acid composition but are clearly divided into two groups: alpha-like chains (α- and ζ-chains) containing 141 amino acids and beta-like chains (β-, γ-, δ- and ε-chains) containing 146 amino acids. Each chain binds a haem molecule, hence there are 4 haem molecules for each Hb molecule called tetramer. As mentioned earlier, sickle haemoglobin (HbS) is the result of a single point mutation of β-globin gene. The mutant gene produces β^S-globin. Therefore HbS is composed of 2α-chains and 2β^S-chains. Diagram of HbS tetramer is shown in Figure 1.3.1.

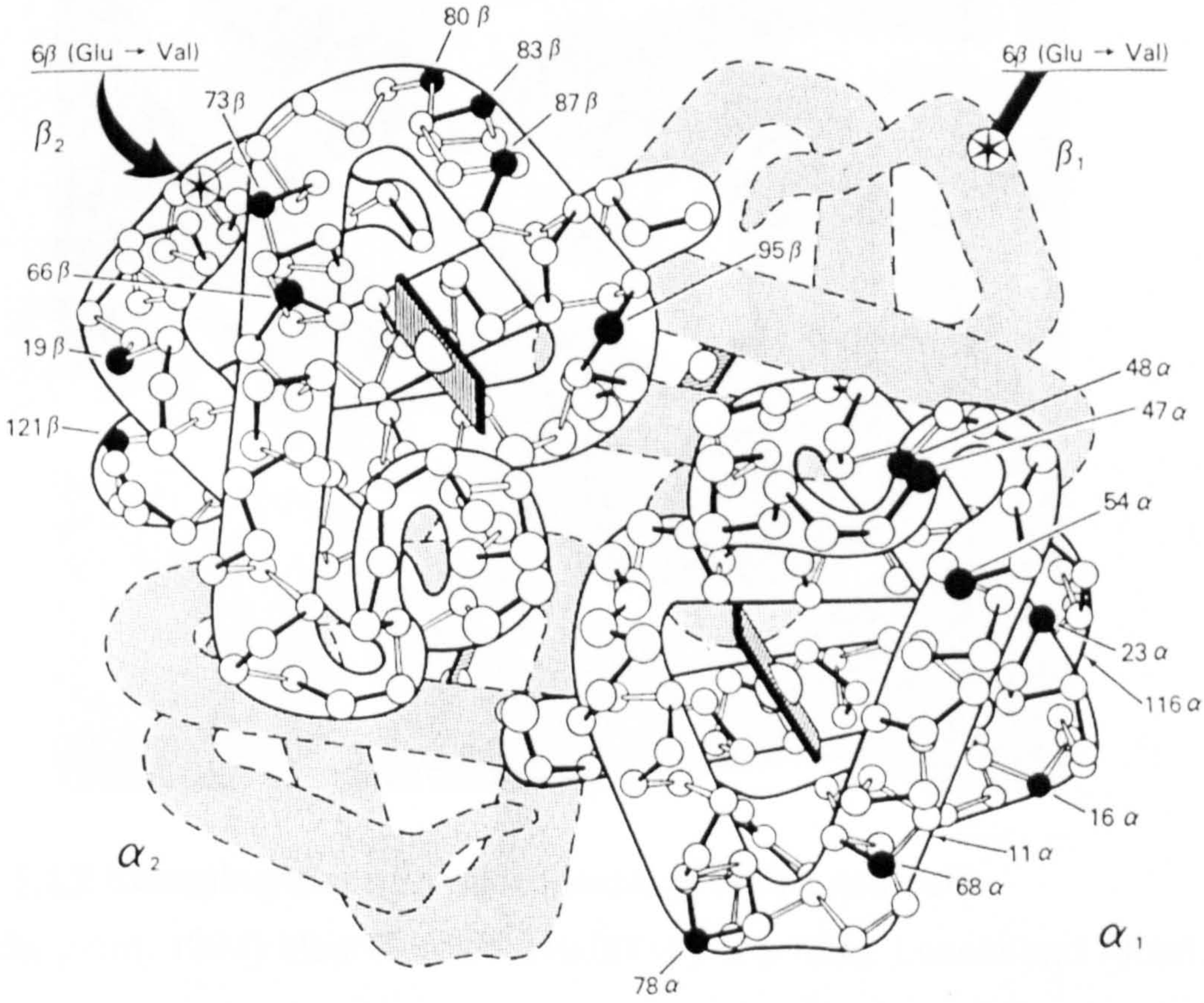


Figure 1.3.1 Diagram of sickle haemoglobin tetramer

(From Dean & Schechter, 1978) The helical outline of each chain and the α-carbon polypeptide backbone of one α- and one β-chain are shown. The β^6 Glu→Val substitutions are indicated with a heavy arrow and a heavy line. Amino acid residues in the region of probable contacts within the polymer are denoted with a filled circle and their number in the amino acid sequence. Arrows indicate residues on the backside of the molecule. The diagram has been simplified in that the identification of all the mutants is shown in the $\alpha_2\beta_2^S$ tetramer whereas their exact location is still ambiguous.

Sickle haemoglobin polymerisation leads to increase cell rigidity, membrane damage and a shape change to crescent or sickle forms (Figure 1.3.2). This provokes many kinds of clinical manifestations, such as vaso-occlusive painful crisis, acute chest syndromes, priapism, stroke, haemolysis and anaemia (Rodgers, 1998).

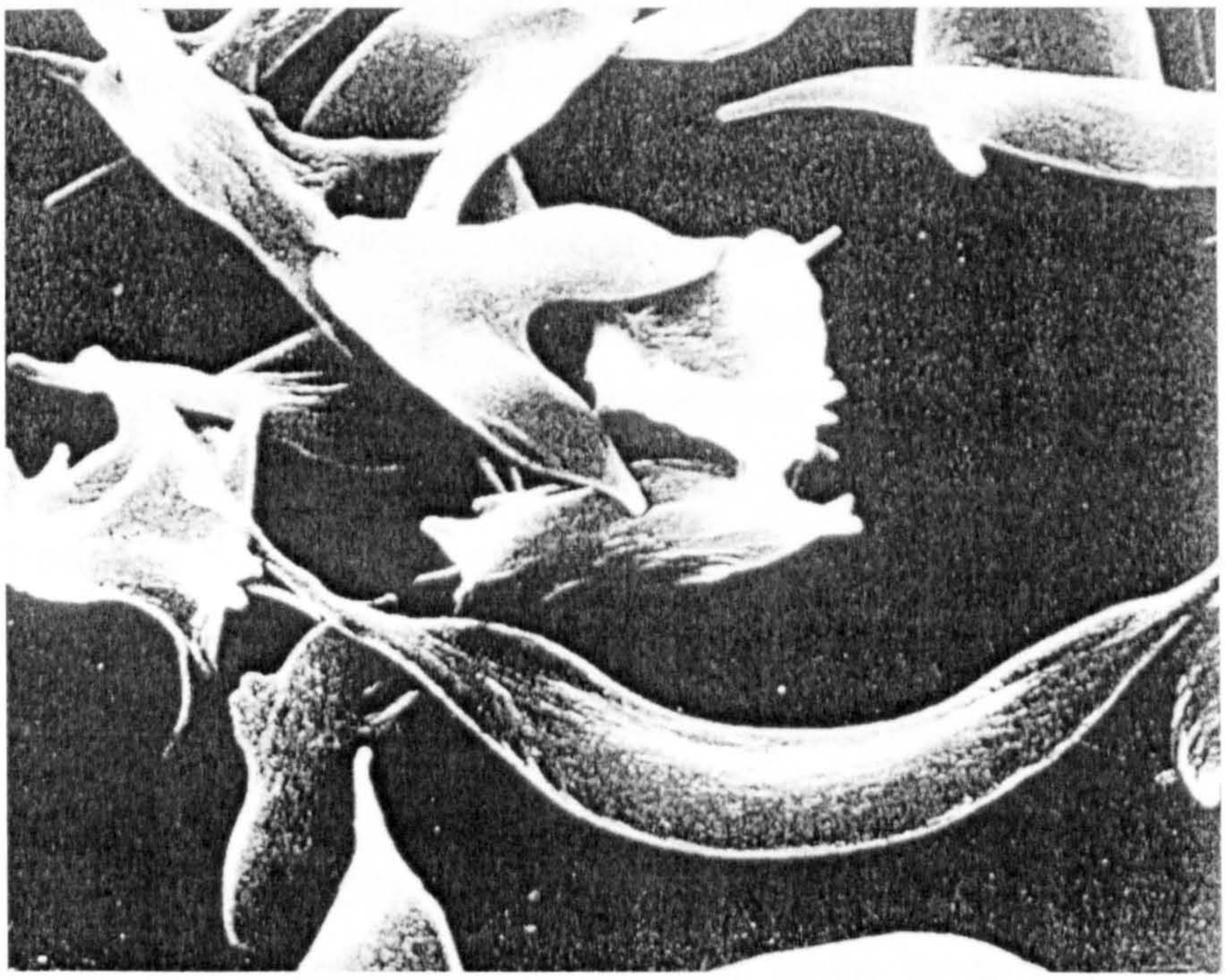


Figure 1.3.2 Scanning electron micrograph of sickle red cells

(From Serjeant, 1994) Magnification x19,000 by Drs Klug, Lessin and Albert.

1.4. GEOGRAPHICAL DISTRIBUTION OF SICKLE CELL DISORDERS

Sickle cell disease is widely distributed among black people and also people in Southern Italy, Northern Greece, Southern Turkey, North-Eastern Saudi Arabia and India. In Africa, clear tribal variations occur between peoples living in similar environments in Uganda and Tanzania. Bantu-speaking tribes have higher and more consistent frequencies than Hermitic-speaking tribes (Lehmann, 1954). Examination of sickle haplotypes shows at least 5 different haplotypes, suggesting at least five separate occasions on which the HbS mutation has occurred independently and been selected for by environmental pressures (Ashley-Koch *et al*., 2000).

Contribution of malaria to the distribution of the sickle cell gene

It is believed that haemoglobinopathies, including sickle cell disease, provide protection against malaria in many malarial regions of the world and that natural selection is responsible for elevating and maintaining their gene frequencies. The epidemiological and *in vitro* data demonstrate that this principle applies to sickle cell disease (Allison, 1964; Roth *et al.*, 1978). Malarial endemic areas usually co-exist with high prevalence of sickle cell trait. The first observation on malaria and the sickle cell trait was in Northern Zimbabwe (former Rhodesia) where it was found that malarial parasites presented less frequently in blood films of sickle cell trait individuals (Beet, 1947). It was confirmed later that malaria was significantly less common in children with sickle cell trait, and that even those who had malaria usually had less severe complications (Raper, 1956). *Plasmodium falciparum* is the only type of malaria that sickle cell trait clearly protects against while protection in other types is inconclusive (Fleming *et al.*, 1979). There is evidence that *P. falciparum*-infected red cells in sickle cell trait patients are sequestrated by the reticulo-endothelial system and exposed to low oxygen tension permitting potassium loss and lowering of pH, factors known to inhibit parasite growth *in vitro* (Ginsburg *et al.*, 1986).

1.5. PATHOPHYSIOLOGY OF SICKLE CELL DISORDERS

1.5.1. Sickle haemoglobin polymerisation

Sickle haemoglobin polymer structure

Polymers of deoxy HbS form fibres termed tactoids. These fibres are composed of up to 14 strands of HbS molecules (Carragher *et al.*, 1988) (Figure 1.5.1.1). In the deoxy HbS crystal, the β^6 valine interacts with the β^{85} phenylalanine and the β^{88} leucine of an adjacent tetramer of HbS (Noguchi *et al.*, 1985) (Figure 1.5.1.2). Other contacts between tetramers occur and are important for polymer

stability, but such contacts alone are not critical for initiation of polymerisation. Other mutations at some of these contact points can stabilise or destabilise the sickle polymer (Watowich *et al.*, 1993).

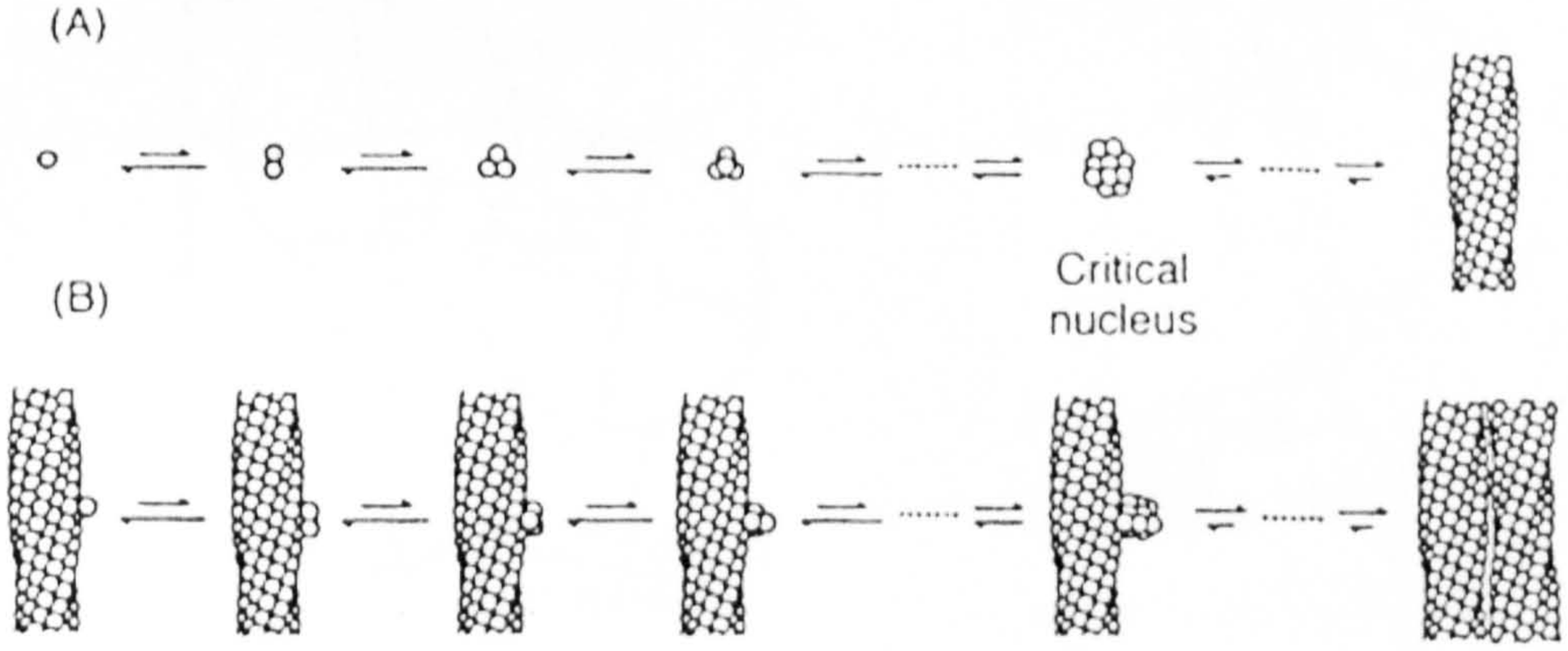


Figure 1.5.1.1 Sickle haemoglobin polymerisation

(From Ferrone *et al.*, 1985)

(A) In homogeneous nucleation of HbS tetramers, a critical nucleus of haemoglobin molecules forms with subsequent appearance of polymer. Polymer formation is not stable until a critical nucleus is achieved.
(B) In heterogeneous nucleation, the additional polymers can grow on the surface of the original polymer causing them to become more stable.

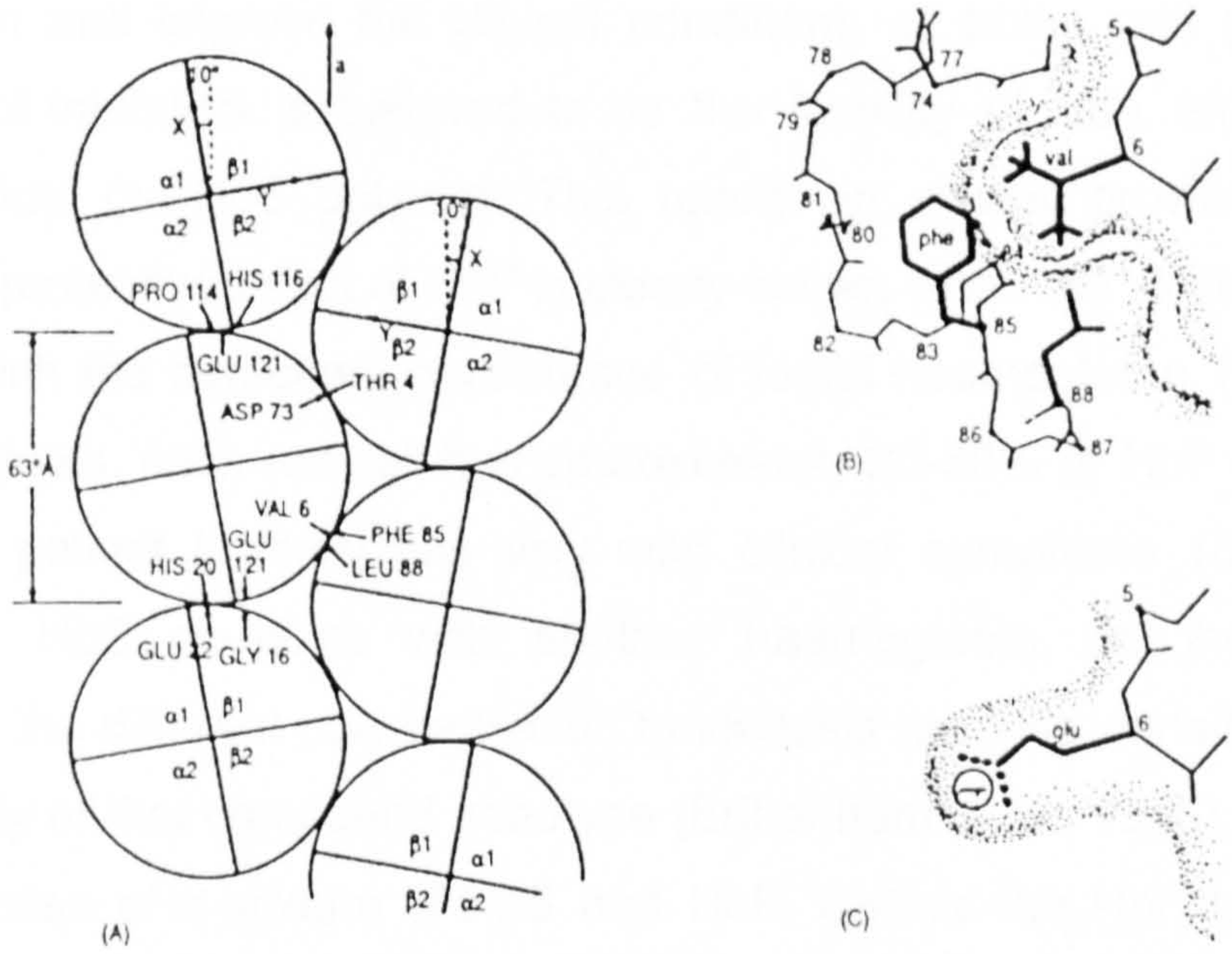


Figure 1.5.1.2 Deoxygenated sickle haemoglobin polymers

(From Ferrone *et al.*, 1985) Contact points are shown. Figures A and B show a single β^S-globin chain, β^6 valine interacting with β^{85} phenylalanine and β^{88} leucine of an adjacent tetramer. In figure C, normal β^6 glutamic acid in HbA cannot make this contact as its charge and size does not fit.

Factors affecting the rate of polymerisation

Polymerisation of HbS in solution is dependent on temperature (Anderson & Biro, 1994), ionic strength (Bookchin & Balazs, 1986), level of 2,3-diphosphoglycerate (Cohen-Solal *et al.*, 1998) and oxygen concentration (Alston *et al.*, 1984). The HbS tetramer concentration defines the solubility of HbS and is in equilibrium with the HbS polymers. Fully oxygenated HbS cannot polymerise, but partially or fully deoxygenated HbS can. The rate of tactoid formation is inverted proportional to the 10^{th} power of the deoxy HbS concentration (Ferrone *et al.*, 1985). Any factor, which increases this concentration, will sharply increase the rate of sickling. For example, dehydration of the red cell increases the haemoglobin concentration and hence that of deoxy HbS, thus increasing the rate of tactoid formation. Conversely factors diluting the concentration of HbS, such as the presence of other normal Hb (HbA or HbF) which interact less rapidly

with deoxy HbS, will slow this process. Thus either HbA or HbF can inhibit HbS polymerisation and improve the clinical conditions of sickle cell patients. The mechanism of inhibition is believed to be the inability of HbA and HbF to be incorporated into the HbS polymer. This results in slower growth of the HbS polymer. The protective effect of HbF is clearly shown when the sickle cell gene is co-inherited with the hereditary persistence of foetal haemoglobin (HPFH) gene. In such individuals, each red cell has approximately 20-30% of HbF in addition to HbS and the patient typically has very mild clinical symptoms (Danish *et al.*, 1989). When HbS co-exists with another haemoglobin, in the compound heterozygote, the different polymerisation tendencies are also consistent with the clinical severity of that compound genotype (Brittenham *et al.*, 1985).

In the case of a mixture of HbS and HbF, neither the HbF tetramer ($\alpha_2\gamma_2$) nor the HbS-HbF hybrid tetramer ($\alpha_2\beta^S\gamma$) can initiate polymerisation. In contrast, the HbS-HbA hybrid tetramer ($\alpha_2\beta^S\beta^A$) has a greater chance of initiation. When a polymer free HbS solution is deoxygenated, a delay time occurs before any polymer is detected and then it rapidly accumulates. This observation is explained by the "double-nucleation" hypothesis in which it is believed that an initial homogeneous nucleation of polymer takes place first in the solution and then a subsequent explosive heterogeneous nucleation on the template of ready-formed polymers occurs later (Eaton & Hofrichter, 1990) (Figure 1.5.1.1). The delay time of HbS polymerisation is inversely correlated to 10^{10} times the concentration of deoxy HbS . This implies that a small reduction in HbS concentration might have a critical role in improving the clinical disease by prolonging the delay time which allows more time for sickle red cells to escape the sickling process at the post-capillary venules (Cao & Ferrone, 1996). The polymerisation inside sickle red cells appears to occur similarly to that of HbS in solution. However, a wide range of different densities and HbS concentrations exists in each patient. This results in a wide range of delay times from milliseconds to many seconds (Noguchi *et al.*, 1983). The variability in delay times determines the different fates of sickle red cells (Mozzarelli *et al.*, 1987) (Figure 1.5.1.3).

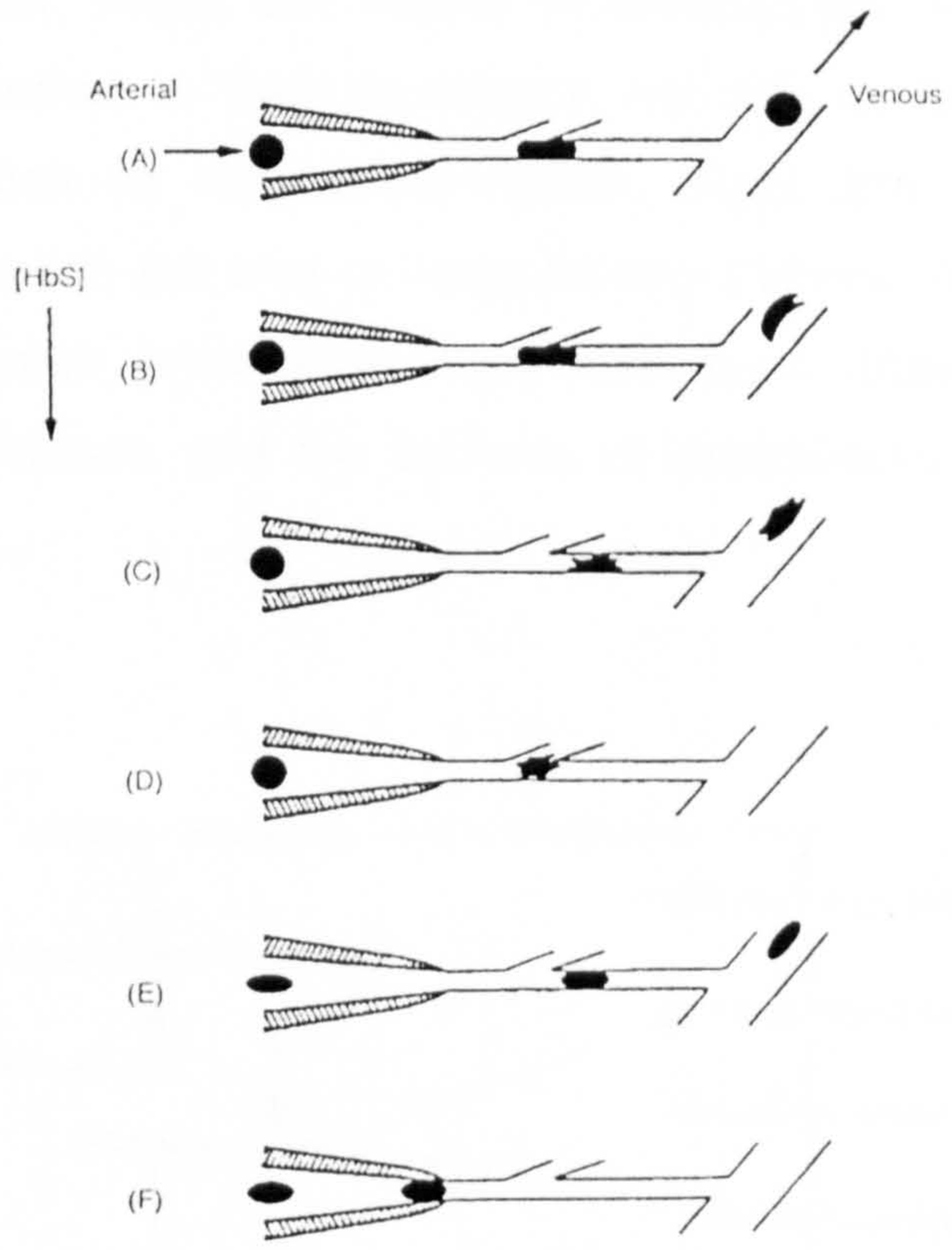


Figure 1.5.1.3 Vaso-occlusion in sickle cell disorders

(From Mozzarelli *et al.*, 1987)

(A) A sickle red cell without polymer or no significant membrane damage, perhaps a HbF containing red cell, is passing through the capillary smoothly.

(B) A sickle red cell with few polymers is trapped for a while, because of its longer delay time, so it escapes the capillary but deforms later in the venule.

(C) A sickle red cell with some polymers is trapped longer, because of its shorter delay time, so it deforms within the capillary but escapes into the venule without any occlusion.

(D) A sickle red cell with more polymers is trapped and obstructs the capillary microcirculation.

(E) A sickle red cell, which is trapped in the capillary, may escape later but goes out with permanent membrane damage.

(F) A sickle red cell with permanent membrane damage and high rigidity is trapped in the microcirculation and produces vaso-occlusion.

These fates of red cells are dependent on the delay time of individual cells, polymer concentration, degree of membrane damage, vascular tone (vasoconstriction and vasodilatation) red cell and endothelial adhesion molecules and plasma factors.

Thus the interaction of any particular sickle red cell and its environment determines whether, when, and where vaso-occlusion will occur. The starting point of vaso-occlusion is likely to depend not only on features intrinsic to the sickle red cell, such as HbS concentration, delay time, polymer content and membrane impairment, but also on local factors extrinsic to the cell, such as the activations of vascular endothelial cells, leukocytes, platelets and coagulation factors, cytokine release and the balance of vasodilators and vasoconstrictors (Figure 1.5.1.4).

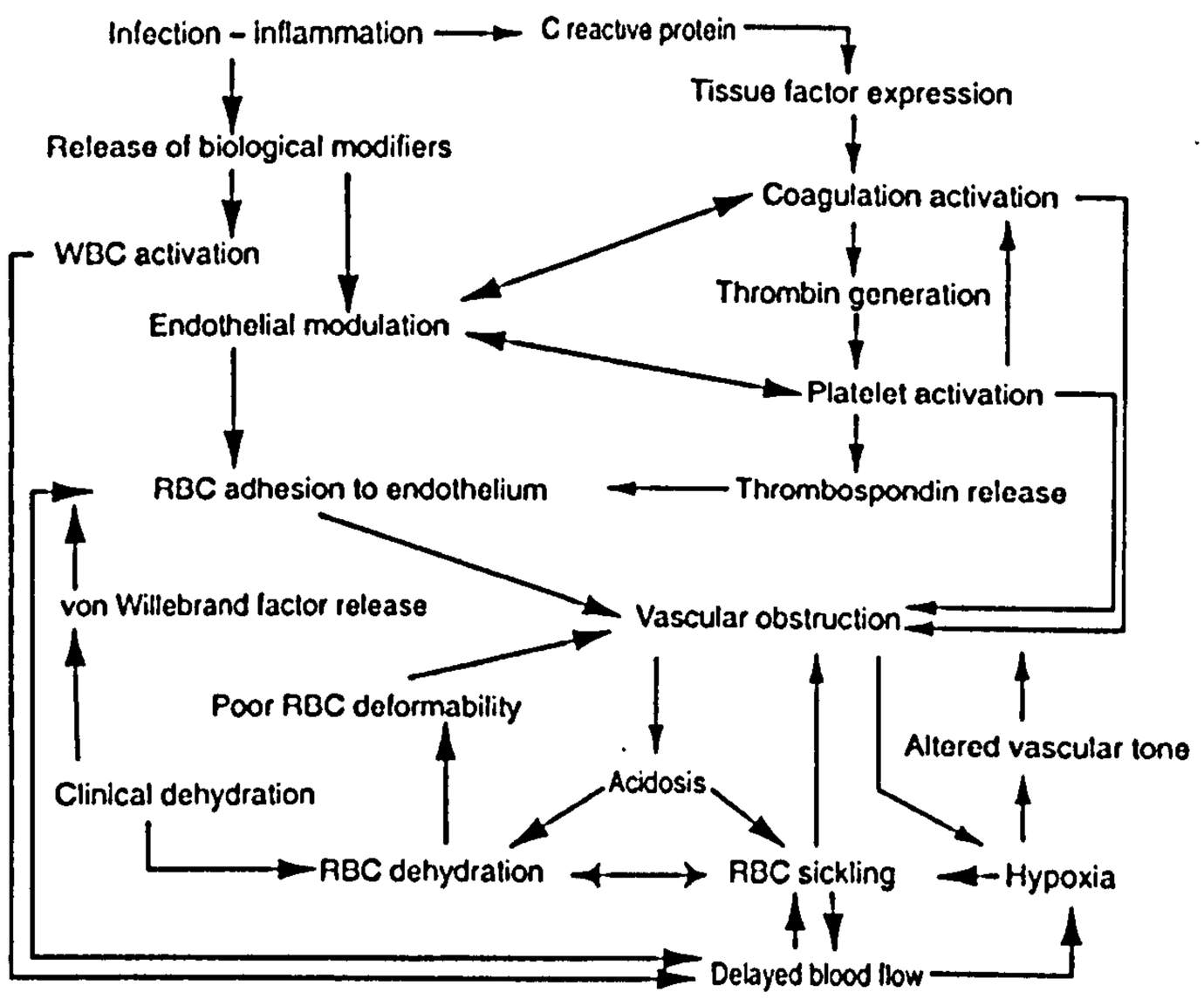


Figure 1.5.1.4 Pathophysiology of vaso-occlusion

(From Embury, 1996) See text for details.

1.5.2. Mechanism of vaso-occlusion

Vaso-occlusion is a multi-factorial process involving many cell types, cytokines, activation markers and microvascular environment (Hillery, 1998). Where ever vaso-occlusion takes place will depend on a balance between the rate of tactoid formation and the rate of blood flow in the microvasculature. Vaso-occlusion is thought to occur predominantly in the post-capillary venules. As the flow slows in post-capillary venules where the pO_2 is low, conditions favouring tactoid formation can arise. The flow rate can be affected by many factors such as the viscosity of the blood, the deformability of the red cells, the vessel diameter, and the tendency of red cells or other cells to adhere to the vascular endothelium. In the 'steady state' a dynamic equilibrium exists between the rate of formation of tactoids at low pO_2 and tactoid dissolution as the red cells flow back to more oxygenated environments. This equilibrium can be disturbed by a number of external factors such as hypoxia (e.g. at high altitudes or intense exercise), vasoconstriction (such as in cold weather or emotional excitements), dehydration and acidosis, which increases risk of vaso-occlusion (especially in inflammatory conditions where adhesion molecules on endothelium are upregulated). These mechanisms appear to be temporally different within the same patient, and between different patients. Genetic factors, acquired red cell characteristics, the internal environment of the patients and the behaviour of the different sickle cell genotypes modulate all plasma factors and vascular factors. All of these determine the vaso-occlusive type and severity of the disease (Stuart & Johnson, 1987). Such disease complexity provides many potential targets for therapeutic interaction. (Figure 1.5.2).

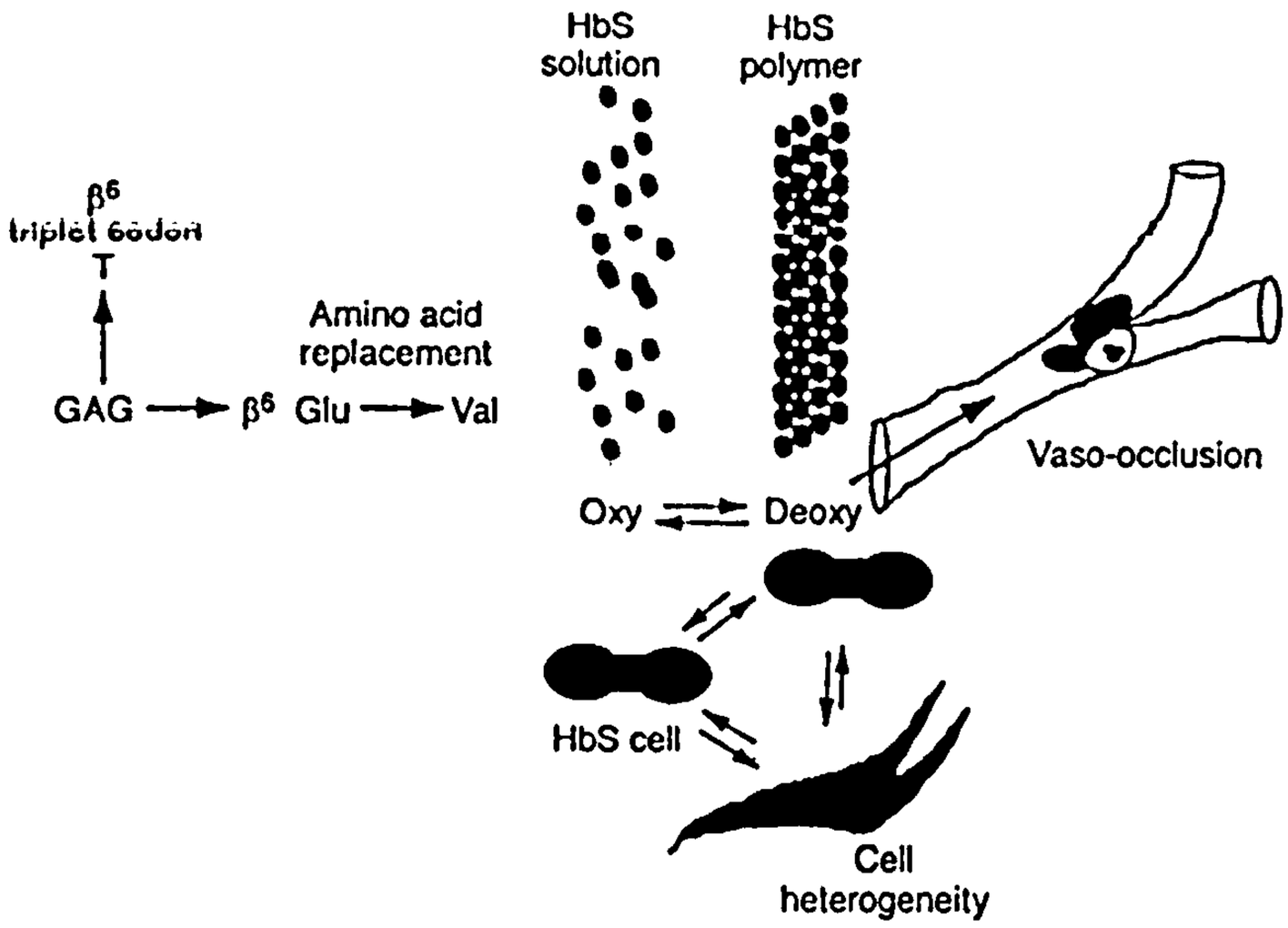


Figure 1.5.2 Pathogenesis of sickle cell disorders

(From Rodgers, 1998) The nucleotide (GAG→GTG) and amino acid (Glu→Val) substitution of sickle haemoglobin (HbS), polymer formation on hypoxia or deoxygenation, cell sickling, cellular heterogeneity, sickle cell endothelial adhesion and vaso-occlusion is shown.

1.5.3. Sickle red cell heterogeneity

The circulating sickle red cells differ even in the same patient (Bertles & Milner, 1968). For example, some sickle reticulocytes are very dense with mean corpuscular haemoglobin concentrations (MCH) of up to 50 g/dl compared with the normal range of 27-38 g/dl. The sickle red cells present in various shapes such as oval, asterisk, crescent or sickle shapes because of the differences in HbS concentrations, rates of polymerisation and content of polymer forms. These include the crenate cells and discocytes, which can be either very dense or normal. Some sickle red cells are irreversibly fixed in their bizarre contour. Some are dense at the time of release from the bone marrow, while others become more dense later in circulation (Bookchin *et al.*, 1991). The density of each sickle red cell is determined by its Hb content and its capacity to maintain normal hydration and ionic balance. The aged cells usually become more dense

because their membrane damage causes ionic imbalance and dehydration (Nash, 1991). They also tend to have more membrane bound IgG (Green *et al.*, 1985). The number of HbF containing red cells (F^+cells) and dense cells can differ widely among patients, and this may be genetically controlled. The degree of haemolysis directly correlates with the number of irreversibly sickle and dense red cells. There is a paradox in the relationship between cell density and vaso-occlusion. The higher proportion of dense cells, the fewer vaso-occlusive painful crises are found (Lande *et al.*, 1988). The proportion of dense cells falls as the crisis starts and rises again as the painful crisis resolves (Ballas & Castillo, 1992). These findings probably suggest that the more deformed, more adherent but less dense sickle red cells may stick to the endothelium inside the vessel wall first, then the dense cells follow and are removed later.

1.5.4. Membrane damage of sickle red cells

Vaso-occlusion is rarely found in sickle cell trait (AS) since each red cell contains only about 40% HbS, the rest being HbA. However, the renal medulla is always acidic, hypoxic and hyper-osmotic. These conditions are suitable for HbS polymerisation. Even sickle cell trait red cells deform in this environment occasionally leading to haematuria. Renal concentrating ability is nearly normal in individuals with sickle cell trait and sickle cell co-inherited with α-thalassaemia (S/α-thal), where the HbS content is about 30% and fewer polymers exist inside the cells. This indicates the importance of the HbS concentration (Gupta *et al.*, 1991). Deoxy-HbS polymer, and perhaps a high concentration of oxy-HbS, may cause secondary damage to the cell contents and membrane. This damage reduces the capacity of sickle red cells to maintain their normal K^+ gradients, which are mediated by activation of the K:Cl co-transport channels, leading to cell swelling, deoxygenation, acidification, high intracellular Ca^{2+} and sickling. Membrane phospholipids may lose their asymmetry by exposing the procoagulant aminophospholipid, phosphatidyl serine (PS), which normally presents inside the cell membrane (Helley *et al.*, 1996). This leads to an inappropriate initiation of coagulation process. Abnormal interactions among

membrane cytoskeletal proteins are also found. This may be due to iron-mediated oxidation, which promotes the clustering of ankyrin, band 3 and glycophorin together with oxidised HbS (Corbett & Golan, 1993). Micro-vesiculation may occur when the HbS polymer penetrates the cytoskeleton and protrudes outwards as a lipid-ensheathed bud, or even dissociates into the circulation as a micro fragment. A relatively rapid reduction of oxygen concentration (50% reduction of O_2 within 1 minute) over sickle red cells causes a sharp increase in individual viscosity and a granular appearance characterized by an irregular surface with small spicule-like projections (Kaul & Xue, 1991). (Figure 1.5.4)

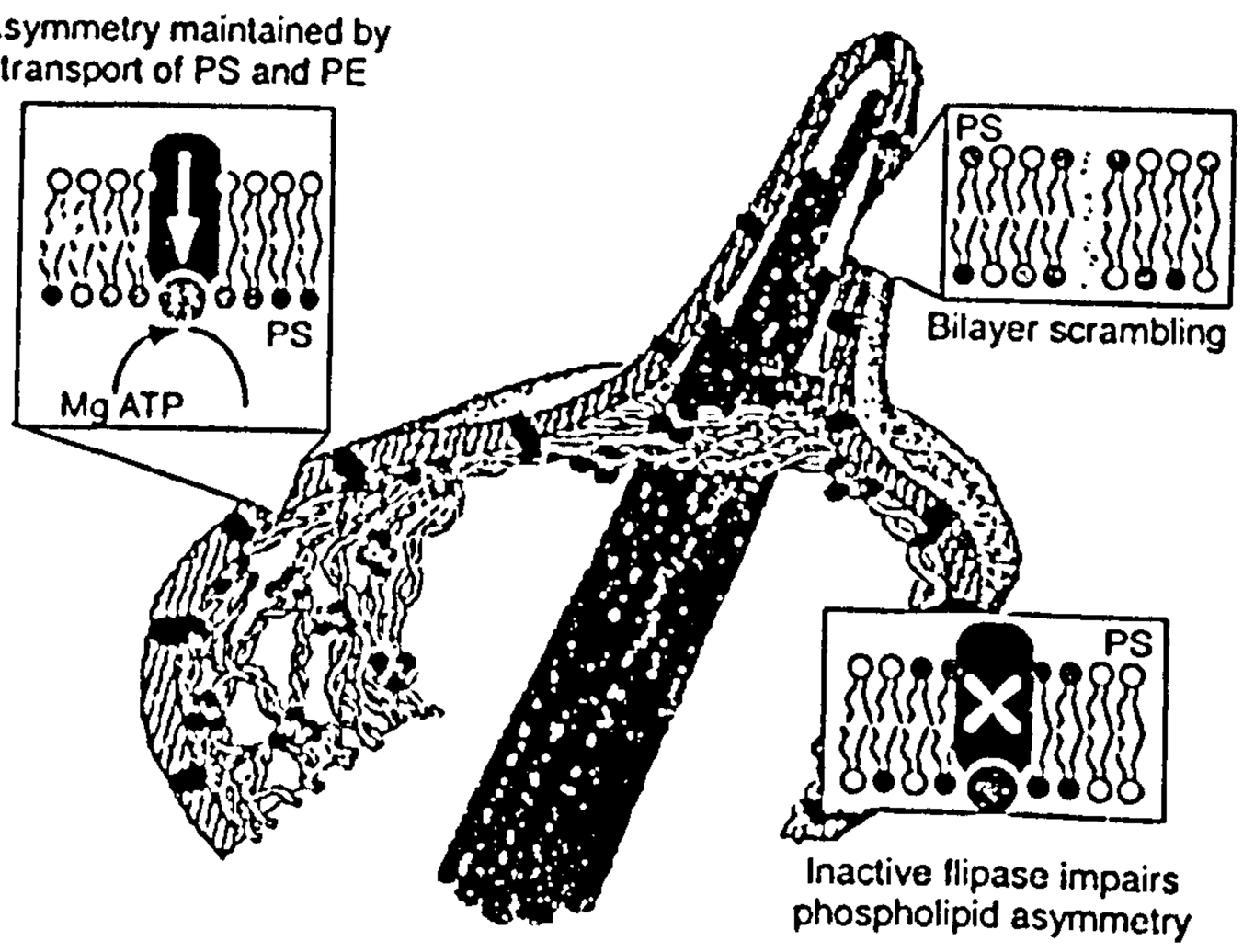


Figure 1.5.4 Membrane damage in sickle red cell

(From Rodgers, 1998) Phosphatidyl serine (PS) and phosphatidyl ethanolamine (PE) are translocated to the outer leaflet of the membrane of sickle red cells, perhaps because of sickling-induced damage and because the enzyme responsible for maintaining normal phospholipid asymmetry is not functioning properly. Sickle haemoglobin polymer protrudes through the membrane skeleton, distorting the lipid bilayer and leading to loss of membrane lipid as micro-vesicles.

Membrane damaged sickle red cells are able to adhere to vascular endothelial cells (Hebbel *et al.*, 1980a). The mechanism of attachment may vary according to the type of endothelium encountered. The adhesion of sickle red cells onto vascular endothelial cells is thought to reduce blood flow so that polymerisation, sickling and vaso-occlusion occur before their transit through the microvasculature is completed. Sickle red cells adhere to vascular endothelial cells via membrane adhesion molecules such as CD36 (GPIV, GPIIIb or thrombospondin receptor), CD41 (GPIIb or αIIb integrin) and CD49d (α_4 integrin) on sickle red cell surface. Some plasma proteins such as von Willebrand factor (vWF), thrombospondin (TSP), fibrinogen (FB) and fibronectin (FN) are thought to bridge the interaction of sickle red cells with vascular endothelial cell surface molecules such as laminin (LM), glycoprotein Ib (GPIb), integrins, vascular cell adhesion molecule (VCAM) and Fc receptor (Fc-R) (Hebbel, 1997; Moore *et al.*, 1996). Clinical severity is thought to be related to the degree of adhesion of sickle red cells to vascular endothelial cells (Hebbel *et al.*, 1980b).

1.6. CLINICAL CONSEQUENCES OF SICKLE CELL DISORDERS

1.6.1. Life expectancy and causes of death in sickle cell disease

Life expectancy varies depending on socio-economic conditions as well as the genotypes of sickle cell disorders. In homozygous sickle cell patients (SS), after the first 6 months of life where persistence of HbF protects against sickling, the highest mortality occurs within the first 5 years with the greatest risk between 6-12 months. In the Jamaican cohort study, there was about 6% mortality within the first year of life, with a continuing but lesser mortality rate thereafter and a 10-year survival of 84% (Thomas *et al.*, 1982). In addition, another peak of mortality appears in women aged between 20-24 years, partly due to pregnancy related complications (Smith *et al.*, 1996). In the USA and the Caribbean, survival of SS patients of Western African origin commonly exceeds

30-40 years. Survival is even greater in SS patients in India and Eastern Saudi Arabia where almost all patients survive into adult life (Gelpi, 1979).

The common causes of death among sickle cell patients are acute chest syndromes, acute splenic sequestration, renal failure and meningitis (Thomas *et al.*, 1982). In infancy, pneumococcol septicaemia predominated in the past, but penicillin prophylaxis has reduced this cause of death (Day *et al.*, 1992). However, penicillin-resistant pneumococcus increasingly threatens the success of this drug (Woods *et al.*, 1997). Renal failure as a cause of death predominates in patients over the age of 40 years. In another study of 3764 American patients, the median age at death was 42 years for male SS and 48 years for female SS patients. Among those with sickle cell co-inherited with HbC (SC) patients, the median age was 60 years for males and 68 years for females. The factors associated with high risk of early death were acute chest syndromes, renal failure, seizures, a base line white-cell count higher than 15,000 cell/mm^3 and a low HbF level. However, over 50% of followed patients survived beyond 50 years (Platt *et al.*, 1994).

1.6.2. Acute complications of sickle cell disorders

Vaso-occlusive painful crisis

Acute vaso-occlusive painful crisis is the most common complaint of patients with SS. The pathogenesis of vaso-occlusion has been described in terms of chaos theory (Embury, 1996). Where the crisis begins is still not clearly known. It is likely to be somewhere in the microvasculature through mechanisms described above, but the larger arteries, especially in the brain and lung, can also be included. Typically painful crises involve the long bones in adult life causing severe limb pain. The ribs may also be involved causing chest pain. In infancy the hands and feet are often involved causing 'hand-foot' syndrome often associated with swelling of these extremities. Experimental evidence suggests that many different elements inside and on the membrane of the sickle red cells, on endothelial cells and in the plasma, provoke and mediate vaso-occlusion.

The frequency and severity vary among patients and also within the same patient from time to time. Any type of stress may precipitate a painful episode. The frequency of painful crises varies directly with the haematocrit but inversely with HbF levels.

Acute chest syndromes

Acute chest syndromes (ACS) are characterised by chest pain, fever, hypoxia, dyspnoea and new infiltrates on chest radiography. ACS is the most common cause of death in adults and the second most common cause of hospitalisation (Vichinsky, 1991). The incidence of ACS is age and genotype dependent. It is 3 times more common in young children than in adults and is most common in SS followed by S/β^{o}thal, SC and S/β^{+}thal in decreasing order of frequency. The incidence of ACS is inversely related to HbF levels and the degree of anaemia, but is directly related to WBC count during the steady state (Castro *et al.*, 1994). ACS is closely associated with vaso-occlusive painful crises especially in adults (Styles *et al.*, 1996). Thirty % of ACS is associated with painful crises. Adhesion of sickle red cells to endothelium of small or even medium pulmonary vessels may be the primary cause of ACS (Haynes & Kirkpatrick, 1993; Sugihara *et al.*, 1992). Pulmonary thrombo-embolism is uncommon as a cause of ACS despite the presence of hypercoagulability in sickle cell patients (Haupt *et al.*, 1982). However after occlusion has occurred, platelet activation, endothelial activation and hypercoagulability are the causes of inflammatory activation. Management of ACS including antibiotics, oxygen inhalation, and transfusion in mild cases and exchange transfusion in severe cases are recommended.

Infection (Pneumococcal septicaemia)

Pneumococcal infection is a high risk in children with sickle cell anaemia (Samuels-Reid, 1984), the greatest risk being in SS children with splenomegaly (Topley *et al.*, 1982) and age under 6 years (John *et al.*, 1984). Pneumococcal pneumonia, septicaemia and meningitis are common and have a high mortality

rate among SS children. Prophylactic penicillin is recommended to start from 4-6 months for entire life. Pneumococcal vaccine should be given at 2 years and a booster dose at 4 or 5 years (Salamah *et al.*, 1987). Other infections are also increased, such as *Haemophilus influenza* type B, salmonella osteomyelitis and *Escherichia coli* septicaemia.

Folate deficiency

Megaloblastic anaemia due to folate deficiency may be found in sickle cell disease associated with severe diarrhoea and low folate levels (Sinow *et al.*, 1987). Most patients respond to folate supplements (1 mg daily) quite well, but the megaloblastic picture may still remain until folate levels are normalised (Rabb *et al.*, 1983). Factors affecting folate deficiency are a low folate diet, diarrhoea, and folic acid antagonists such as pyrimethamine and anticonvulsant drugs. In sickle cell disorders, the haemolytic process makes increased folate demands. Folate supplements (1-5 mg daily) is recommended for children with sickle cell disease.

Aplastic crisis

Aplastic crisis is characterised by acute severe anaemia (reduction of Hb from 8.6 to 3.5 g/dl within a few days) with a low reticulocyte count (0.2%) and a marked decrease in red cell precursors in the bone marrow. This can be rapidly fatal if not recognised rapidly. The bone marrow typically recovers within 10 days with intense erythroid hyperplasia, increased reticulocytes and Hb increasing to the previous levels (Morley *et al.*, 1973). The aetiology of the crisis is human parvovirus (B19) infection (Kelleher *et al.*, 1984). This infection occurs in about two thirds of children in the UK but only causes problems if red cell survival is short, such as in SS or other haemolytic disorders. Supportive treatment and blood transfusion are required and prognosis is usually good if the condition is recognised early (Goldstein *et al.*, 1987).

Acute splenic sequestration

Acute splenic sequestration is characterised by a large number of red cells sequestrated within the spleen, leading to a sudden fall in Hb level, usually life-threatening and with high prevalence in young children aged less than 5 years (Topley *et al.*, 1981). Splenic enlargement, a sharp drop in Hb to lower than 2 g/dl within a few days and sudden collapse are common features. Most patients require emergency blood transfusion. Splenectomy for prophylaxis of further episodes is usually recommended after 2 attacks (Kar, 1999).

Stroke

Stroke follows occlusion of cerebral vessels and affects about 10% of patients with SS (French *et al.*, 1997). This occurs most commonly in young children, median age at 6 years, and has a 50-70% chance of recurring within 3 years after the first attack. Chronic transfusion can reduce the risk of a second attack. Trans-cranial Doppler to detect velocity of blood flow or MRI to detect infraction in the cerebral may predict stroke risk (Adams, 2000).

Priapism

Priapism is characterised by congestion of the corpora due to the occlusion preventing drainage of blood out of the penis. Acute priapism is characterised by prolonged painful erection for several hours. Exchange transfusion is required but surgical drainage is often needed. Stuttering priapism is characterised by repetitive, reversible, painful erection usually lasting less than 3 hours and may have a severe attack lasting more than 3-4 hours. Such an episode may cause permanent scarring and/or impotence (Monga *et al.*, 1996).

1.6.3. Chronic complications of sickle cell disorders

Chronic haemolytic anaemia

All sickle red cells do not survive equally because of their cellular heterogeneity. The HbF content of the sickle red cell is the most prominent factor that effects survival, possibly by reducing the tendency towards membrane damage as HbF can delay or prevent HbS polymerisation (Bertles & Milner, 1968). Quite a few sickle red cells are destroyed intravascularly. Most of them have serious membrane damage due to mechanical forces and they are very sensitive to complement-mediated lysis. However, the majority of sickle red cells are destroyed extravascularly. They contain more immunoglobulin G (IgG) on their surface than normal red blood cells. This may be due to the membrane damage caused by both HbS polymerisation and oxidative free radicals. Macrophages, with IgG-Fc receptors, bind these cells and clear them from the circulation. The densest cells appear to have more surface bound IgG, and to be more rapidly sequestered. The degree of haemolysis is directly related to the proportion of IgG bound sickle red cells and/or dense sickle red cells (Green *et al.*, 1985). Sickle red cells entrapped in the microvasculature may be sequestrated rapidly, typically in the spleen. Linking haemolysis to the vaso-occlusive process is the fall in dense cell numbers during the first stage of the painful crisis, which is followed by reticulocytosis, a response to the anaemia resulting from increased haemolysis. Sickle reticulocytes seem to contain more membrane adhesion molecules than normal reticulocytes. Such cells may either initiate or prolong the painful crisis. Once the crisis is resolved, the dense sickle red cells rebound to the level shown before the crisis, probably because there is no more entrapment or sequestration. This explanation may not fit in all crisis patients since the mechanism underlying vaso-occlusive painful crises is very complicated and many points remain to be explored. Increased haemolysis alone is not enough to be the major cause of clinically significant worsening of the anaemia in sickle cell disease (Mohandas & Evans, 1989).

The lifespan of sickle red cells is decreased from a normal 120 days to just 10-20 days in most patients. Haemoglobin levels are typically about 6-9 g/dl and the reticulocyte count is about 5-15%. In the steady state, tissue oxygen delivery is nearly normal because of the hyperdynamic circulation and much lower oxygen affinity of HbS. Persistent anaemia is due to the higher turnover rate of red cells and haemolysis. Extravascular haemolysis causes increases in bilirubin levels which may be associated with jaundice and gall stones especially in adult patients over the age of 20 years (Ballas *et al.*, 1980).

Avascular necrosis

Avascular necrosis or osteonecrosis may occur in sickle cell patients, especially in the femoral head (Hernigou *et al.*, 1993). This complication is more common in SC than SS possibly because of the higher blood viscosity in SC (Ballas *et al.*, 1989).

Chronic renal failure

Chronic renal failure (CRF) is often found in sickle cell patients over age of 40 years and is a serious condition (Wong *et al.*, 1996). Patients at this age should have their renal function monitored regularly. Falling of Hb levels and worsening anaemia with impaired renal function are the signs of CRF. Blood transfusion may be necessary to improve oxygen delivery. In case of end stage renal failure, haemodialysis is required (Powars *et al.*, 1991). Renal transplantation can be considered if the organ is available (Ojo *et al.*, 1999).

Retinopathy

Proliferative sickle retinopathy (PSR) is described as arterio-venous communication lesions at the border of the vascular and avascular retina (Mason & Scherrer, 1991). Complications include retinal haemorrhage and retinal detachment (Siegel, 1988). PSR is more common in SC disease and S/β^+ thalassaemia type III than in SS (Talbot *et al.*, 1988). Males are more affected than females. Peak PSR prevalence in SS is 30 years but in SC is 25-29 years in

males and over 40 years in females. Vitrectomy and retinal reattachment surgery are necessary in severe cases (Penman & Serjeant, 1992).

Chronic leg ulcers

Chronic leg ulceration is a major cause of morbidity and commonly found in adolescence and early adult life (Baum *et al.*, 1987). Osteomyelitis may complicate deep ulcers. Supportive treatments are required along with improved personal hygiene (Sher & Olivieri, 1994). Blood transfusion, exchange transfusion and hydroxyurea treatment may benefit.

1.6.4. Management of sickle cell disorders

Prevention of infections

Sickle cell patients (SS) has an unusual relationship with certain infections. Individuals with sickle cell trait (AS) are less likely to die following a first infection with *Plasmodium falciparum* than normal (AA) subjects, but in SS such an infection may precipitate a fatal crisis. Several acquired abnormalities render SS more susceptible to a number of infections (Fleming, 1989; John *et al.*, 1984). The increased susceptibility to infection with polysaccharide-encapsulated bacteria (*S. pneumonia* and *H. influenzae*) is secondary to the absence or impairment of splenic function. Thus, all SS patients should receive the polyvalent anti-pneumococcal vaccine every 3-5 years and *Haemophilus influenza* type B vaccine at 2, 4 and 6 months after birth. Other recommended vaccinations, in addition to routine childhood vaccines, include influenza vaccine annually and hepatitis B vaccine at birth or first visit of children and adults who have no serological evidence of previous exposure to hepatitis B virus. Prophylactic oral penicillin should be given to infants and children with SS on a daily basis for a minimum of 5 years or for life (Gaston *et al.*, 1986). Cellular immunity may be compromised by transfusion-related iron overload and abnormalities in B-cell immunity may explain antigen-processing defects. *Escherichia coli* infection is usually associated with urinary tract infections in

adult females. Patients with SS are also susceptible to osteomyelitis secondary to *Salmonella typhimurium* in addition to the usual causes of bacterial osteomyelitis, such as *S. aureus*.

Role of blood transfusion

Most SS patients tolerate chronic anaemia well, because HbS has decreased oxygen affinity and is thus more efficient in delivering oxygen to tissues. Moreover there is a direct relation between haematocrit value and painful episodes. Painful episodes occur more frequently in mild rather than severely anaemic patients. A higher blood viscosity may worsen the severity and increase the frequency of vaso-occlusive painful crises.

Transfusion should be considered carefully because an increase in blood viscosity can precipitate a crisis. There are two independent objectives of blood transfusion in SS patients. One is the improvement of oxygen-carrying capacity in severely anaemic patients. The second is a dilution in the proportion of circulating sickle red cells in order to improve microvascular perfusion, such as in acute chest syndromes or in the prevention of recurrent stroke (Styles & Vichinsky, 1997). Specific indications for blood transfusion are listed in Table 1.6.4.1.

Table 1.6.4.1 Indications for blood transfusion in sickle cell disease

(From Rodgers, 1998)

Accepted indications

Simple transfusion

Hb <5.0 g/dl with significant signs and symptoms of severe anaemia

Angina or high cardiac output

Acute haemorrhage

Acute splenic or liver sequestration

Pre-operative preparation with general anaesthesia

Exchange transfusion

Acute cerebral infarction

Acute chest syndrome

Multi-organ failure syndromes including fat embolism

Acute priapism unresponsive to therapy

Surgery

Prevention of recurrent stroke in children

Possible indications

Leg ulcers

Complicated pregnancy

Chronic organ failure

Before injection of hypertonic contrast media

Adults with cerebrovascular accident history

Frequent painful episodes

The aim of exchange transfusion is to increase or to maintain a Hb level at over 10 g/dl and to decrease HbS level at lower than 30%.

All SS patients older than 6 months should have complete red cell antigen phenotyping prior to the first transfusion and the records kept available at all times in the blood bank. Phenotype determination should include at least ABO,

Rh, Kidd, Kell and Duffy blood groups. Leukocyte-reduced red cell concentrate is recommended especially in children because it reduces febrile non-haemolytic transfusion reaction, alloimmunisation and cytomegalovirus (CMV) infection and minimises reactions to white blood cells. (Rodgers, 1998)

Role of hydroxyurea

It has been recognised for many years that HbF prevents HbS polymerisation and thus reduces the severity of sickle cell disease. Hydroxyurea (HU) has been shown both to increase HbF levels and decrease the severity of the disease (Charache, 1993). At first the clinical benefit of HU was thought to be its action of increasing HbF levels alone, but it appears that clinical benefit is observed before maximal HbF levels are achieved (Styles *et al.*, 1997). Later studies showed that the reduction in neutrophils, monocytes, platelets and reticulocytes due to bone marrow suppression may play a more important role in contributing to the clinical benefit (Charache *et al.*, 1996).

A double-blind placebo-controlled trial of HU in 299 adult SS patients who had had at least 3 painful episodes per year, showed that HU significantly reduced the frequency of painful crises, ACS, hospitalisation and the need for blood transfusion (Charache *et al.*, 1996). The HbS-containing red cells became less dense, and haemolysis was reduced. These changes and the reduction in painful episodes are achieved before the increases in the HbF levels (Styles *et al.*, 1997). In other studies, HU reduced the adherence of sickle cells to endothelium *in vitro* and increased the length of time for HbS polymerisation or delay time (Adragna *et al.*, 1994; Bridges *et al.*, 1996). Reticulocytes expressing adhesion molecules on their surface are reduced in HU treated SS and SC patients (Browne & Hebbel, 1996). These reductions correlated with the reduction in the frequency of painful episodes (Steinberg *et al.*, 1997). Mechanisms of action of hydroxyurea in sickle cell disease are summarised in Figure 1.6.4.

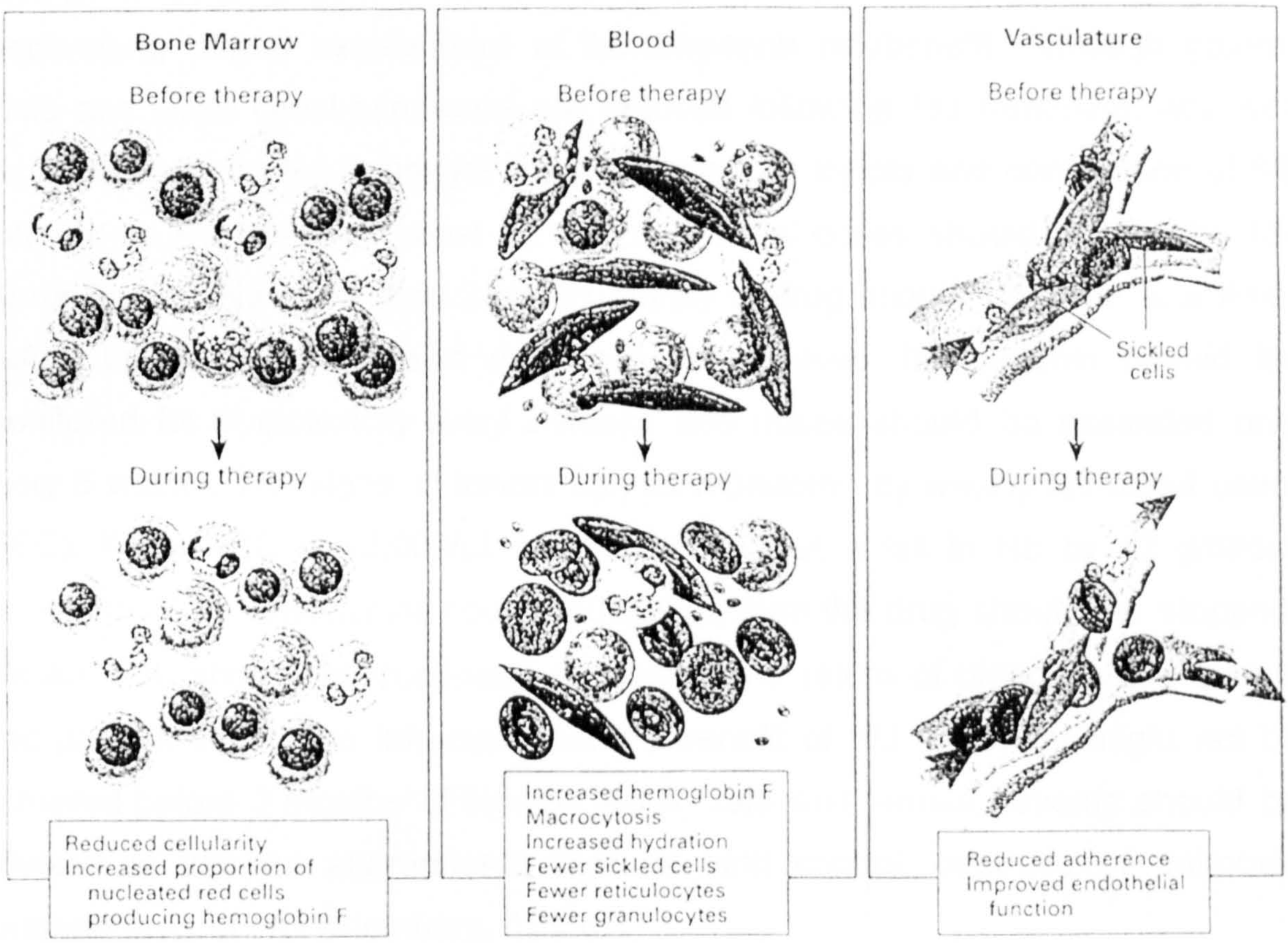


Figure 1.6.4 Mechanisms of action of Hydroxyurea in sickle cell disease

(From Steinberg, 1999) In the marrow, hydroxyurea increases the proportion of erythrocytes containing HbF by its antiproliferative and possibly apoptotic effects on progenitor cells. The proportion of HbF containing cells may be increased because hydroxyurea results in a reduction in the number of cell divisions prior to the release of red cells from the bone marrow. Hydroxyurea has no known direct effect on gene expression. In the bloodstream, higher concentrations of HbF reduce the polymerisation of HbS and the numbers of deformed, dense and damaged red cells. Red cells with a high HbF content survive longer, attenuating haemolysis and leading to reduction in reticulocyte count. The numbers of circulating reticulocytes, granulocytes, monocytes and platelets are also reduced. The likelihood of vaso-occlusion is reduced by the reduction in the number of dense, poorly deformable red cells and adhesion molecule expressing reticulocytes.

There are still many unknowns in HU action. It is unclear whether the clinical benefit results directly from increase in HbF levels or other factors such as decrease in white blood cells, platelets, reticulocytes and red cells expressing adhesion molecules (Steinberg, 1999). Other unknowns include, the optimal

dosing schedules, prediction of toxicity, prediction of responders and non-responders, proper assessment of the long-term risk/benefit. Although painful crisis and acute chest syndrome are reduced following HU treatment, however the impact on survival is not yet known. Monitoring toxicity and compliance of SS patients on HU treatment must be vigorous. Initial doses should begin with 15-20 mg/kg/day. No more than 2 weeks supply of drug should be given at a time. Before the maximal tolerated dose can be achieved, the patients should be monitored for myelotoxicity every 2 weeks and doses should be escalated only every 8 weeks. The signs of toxicity can be monitored by weekly full blood count (FBC). If the WBC is <2,000/μl, platelet <80,000/μl, a fall in Hb by <2 g/dl/day and/or absolute reticulocyte count <80,000/μl, then the drug should be stopped. Weekly FBC should be continued to monitor the return of blood cells. Patients and parents should be informed that the benefit of HU treatment might not be achieved before 3 months of therapy. Adult male and female patients should be advised to use an appropriated form of birth control, because of unknown teratogenesis of HU (Steinberg, 1999).

Bone marrow transplantation

Children age under 16 years with severe complications (stroke, recurrent ACS, refractory pain) and with an HLA-matched sibling donor available, are the best candidates for bone marrow transplantation (BMT). Only a very small number of sickle cell patients have undergone BMT so far. More than 90% of SS patients who have undergone BMT survive, 70-85% had disease-free survival and 15% had graft rejection. Neurological complications (seizures or intracranial bleeding) were common in the first BMT recipients (Walters *et al.*, 1996). Careful control of blood count, blood pressure and anticonvulsant prophylaxis may prevent complications. Follow-up is still short and the full extent of adverse reaction is unknown. The ability of BMT to reverse organ damage is also unknown, but early reports suggest some improvement in lung, bone and CNS. It has been suggested that cord blood stem cells may provide more resources for BMT in a larger number of sickle cell patients (Brichard *et al.*, 1996). As more

beneficial but potentially hazardous treatments as BMT become available, it would be of great value to be able to predict the severity of SS more accurately. Low HbF levels and the absence of α-thalassaemia may predict a more severe clinical course (Thomas *et al.*, 1997). However with modern supportive care, most patients can live longer than 50 years with an acceptable quality of life. BMT may be associated with a short-term mortality of about 10% and this factor should be carefully considered. Controlled trials comparing conventional treatments such as blood transfusion, hydroxyurea therapy and transplantation have not been conducted and therefore the relative values of these treatments are still unclear.

Table 1.6.4.2 Limitations and risks of three main treatments for sickle cell disease (from Vichinsky, 1991)

Blood transfusion	Hydroxyurea Therapy	Bone Marrow Transplantation
Alloimmunisation	Dose variability	Graft rejection
Iron overload	Chronic myelotoxicity	Limitation of donors
Infection	Variable response	Late malignancy
Venous stenosis	Late malignancy	Graft versus host
	Teratogenesis	Central nervous system
	Not proven in children	Sterility/growth impairment
		Short term high cost

Prevention

Prevention of sickle cell disease has been mainly limited to couples living in the developed countries with a high level of education and easy to access genetic services. A couple where both partners have sickle trait have a 1 in 4 chance of having a child with SS in each pregnancy. The foetus can be tested by chorionic villus biopsy at 10 weeks of gestation and a termination offered to the couple if this is culturally acceptable to them. As with other genetic diseases, the prevention programme is based on mass education, screening and genetic counselling. It should be pointed out to a couple that sickle cell disease has a

very variable (and presently unpredictable) clinical course because of genetic and environmental modifying factors. The genetic modifying factors are only partially understood.

Prenatal diagnosis of sickle cell disease

The substitution from glutamate to valine at β^6 position in HbS molecule results from a point mutation in the DNA at that codon from GAG to GTG. This mutation can be detected by cleaving the DNA with a restriction enzyme that recognises the sequence in this region. The Mst-II restriction endonuclease targets the sequence of CCTXAGG (where X is any base) which is present on β^A gene but absent on β^S gene. The digesting on product contains 1.3 kb fragments, because of the missing of a target on β^S gene, corresponding to 1.1 kb fragments in the β^A gene digesting on product. The fragments are then separated by gel electrophoresis and visualised by Southern blotting using ^{32}P-labelled DNA probe, which is complementary to both fragments, because the 1.3 kb fragment also contains the 1.1 kb sequence. An autoradiograph reveals whether the β^A gene, the β^S gene, or both are present in the DNA sample, which was obtained from a chorionic villus biopsy or amniocentesis.

1.7. FOETAL HAEMOGLOBIN AND SICKLE CELL DISORDERS

1.7.1. Foetal haemoglobin levels and distribution

Genetic control of HbF levels

Globin gene switching and sequential gene activation and silencing during development involve complex interactions of erythroid-specific and ubiquitous *trans*-acting sequences that act positively or negatively to regulate transcription (Grosveld *et al*., 1993a). Located in a region of DNA that lies 6-18 kb 5' to the ε-globin gene, and composed of a series of DNase hypersensitive sites (HSs), the locus control region (LCR) plays a critical role in gene expression by

interacting with the promoters of the β-like genes (Grosveld *et al.*, 1993b). Each HS contains genetically conserved binding domains for erythroid-specific and ubiquitous DNA-binding proteins (Figure 1.7.1). The LCR may act as a holocomplex, interacting with only a single promoter at any instant in time (Slightom *et al.*, 1997). An arrangement where the gamma gene was proximal and the beta gene distal to the LCR resulted in predominant gamma-gene expression in the embryo. When the order was reversed and the gamma gene was placed distally to the LCR, gamma-gene expression in the embryo was still up to threefold higher than expression of the LCR-proximal beta gene. These findings suggest that the embryonic trans-acting environment interacts preferentially with the gamma genes irrespective of their order or proximity to the LCR (Peterson & Stamatoyannopoulos, 1993). Erythroid-specific transcription factors have been identified. GATA-1, related zinc-finger binding proteins and Friend of GATA play important roles in erythroid cell development and the transcription of both globin and non-globin erythroid-specific proteins. NF-E2, a B-zip transcription factor, consists of two subunits, one widely expressed and the other restricted to haemopoietic tissues. Erythroid Kruppel-like factor (EKLF) may be the most specific of the known erythroid transcription factors. Interacting with the β-globin gene promoter, EKLF may influence the γ-β switch (Miller & Bieker, 1993) (Perkins *et al.*, 1995).

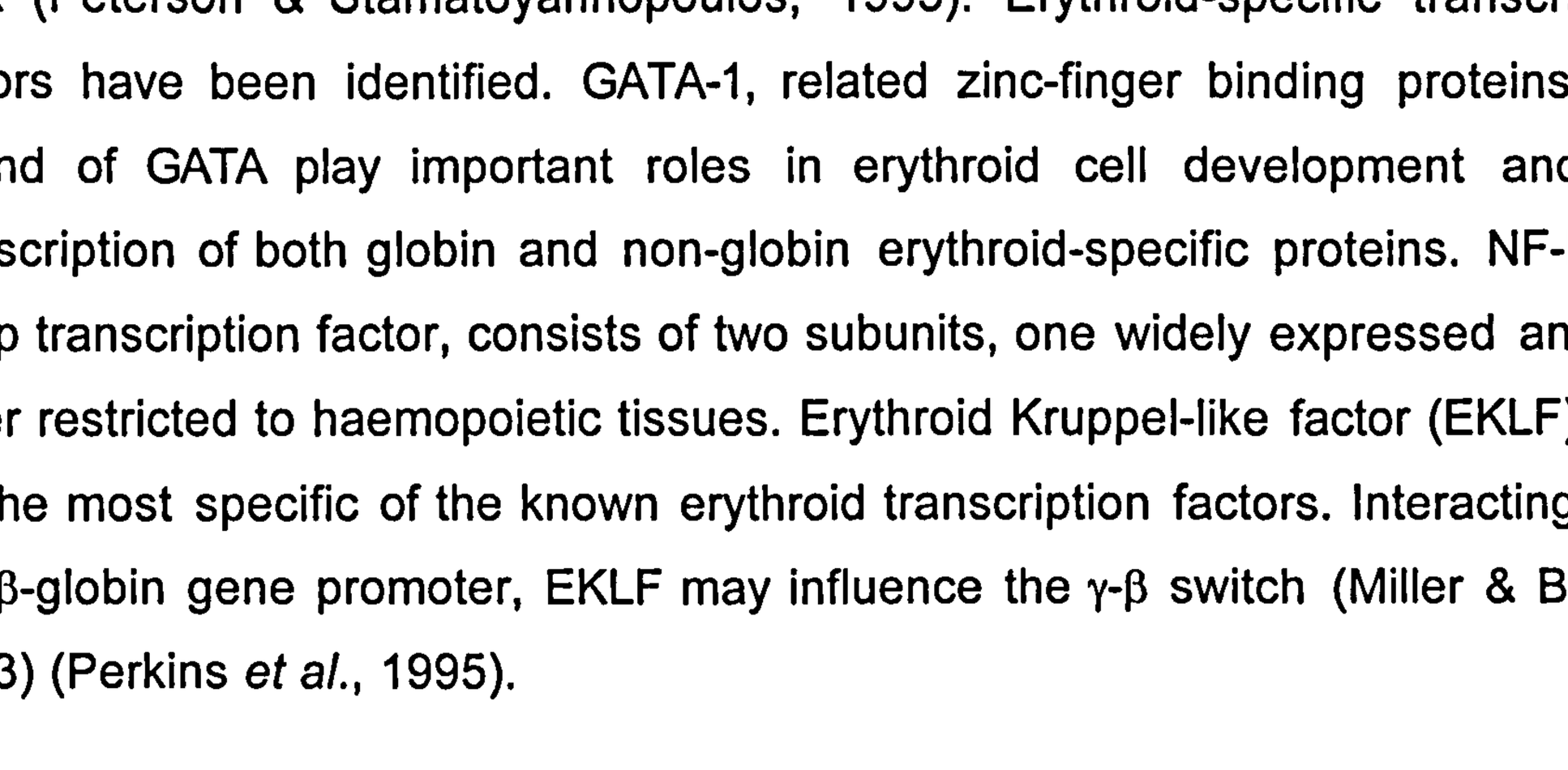


Figure 1.7.1 DNase hypersensitive sites of the β-globin gene cluster

(From Lu & Steinberg, 1996) DNase hypersensitive sites (HSs) of the β-globin gene cluster local control region (LCR) and cis-acting elements that play a role in the modulation of γ-globin gene expression in sickle cell disorders. Within the large arrows are the regions examined in sickle cell patients who had high or low foetal haemoglobin (HbF) levels.

Levels of HbF after birth

After birth the proportion HbF continues to fall during childhood to the adult levels of less than 2% and still declines throughout adult life at a very slow rate. The $^{G}\gamma : {}^{A}\gamma$ ratio varies in adults (Enoki *et al.*, 1990) but $^{G}\gamma$ is predominant in new-born babies (3:1). Thus, the α-globin like genes undergo just a single switch in expression (embryonic→foetal/adult), whereas the β-like genes undergo switches twice (embryonic→foetal→adult). In a small proportion of healthy individuals, HbF levels persist above 1-2% in adult life. The reasons for this are many and complex and are discussed below.

Cellular distribution of HbF

Analysis of the cellular distribution of HbF in normal adults using a sensitive immunofluorescent staining (Wood *et al.*, 1975) shows that only a small subpopulation (1-7%) of red cells contain detectable amounts of HbF (F^{+}cells). However, F^{+}cells in adults are not similar to foetal red cells in new-born babies, neither in the amount of HbF per cell nor in the other characteristics (e.g. surface antigen, red cell enzymes, etc.) (Weatherall, et al. 1981). In healthy adults, the amount of HbF per F^{+}cell ranges up to 25% of total Hb in the cell (Boyer, 1975) and it is now clear that different F^{+}cells have unequal amounts of HbF. In neonates, the proportion of HbF in foetal red cells may range from 50-90% (Weatherall, et al. 1981). The fall in HbF in the first year of life is associated with a fall in both the proportion of F^{+}cells and the HbF content in the F^{+}cells themselves (Henri *et al.*, 1980).

It seems that all red cell precursors have the capability to express some HbF but only a small subpopulation do so in detectable amounts. This concept is supported by findings that in clonal disorders of haemopoiesis such as polycythaemia vera and chronic myeloid leukaemia, the cellular distribution of

HbF or the proportion of F^+cells is still the same as that in normal individuals (Papayannopoulou *et al.*, 1978). If F^+cells represented a distinct cell population, then most of these patients would have no F^+cells at all, while a few would have all F^+cells.

1.7.2. Factors modulating HbF levels in adult life

Genetic factors (Gilman & Huisman, 1984), gender and age (Miyoshi, *et al.*,1988; Maier-Redelsperger, *et al.*, 1994) may effect HbF levels. High HbF levels in adult life are acquired or inherited factors. The acquired factors consist of pregnancy, acute blood loss, leukaemia, bone marrow transplantation, aplastic anaemia, refractory normoblastic anaemia, paroxysmal nocturnal haemoglobinurea, infant having a diabetic mother and juvenile chronic myeloid leukaemia. The inherited factors can be divided into 2 sub-groups; primary and secondary. The primary sub-group consists of hereditary persistent foetal haemoglobin (HPFH) and δβ-thalassaemia that can be divided in to non-deletion and deletion forms. The non-deletion form is further divided into autosomal-linked and X-linked. The secondary sub-group consists of sickle cell anaemia and β-thalassaemia.

Hereditary persistent foetal haemoglobin (HPFH) and δβ-thalassaemia

Hereditary persistent foetal haemoglobin (HPFH) and δβ-thalassaemia are heterogeneous disorders, which are characterised by increased levels of HbF in adult life, because of a decrease or absence of β-chain synthesis and a variable compensatory increase in γ-chain synthesis. The distinction between them was originally made on clinical and haematological grounds before their related molecular defects were recognised.

HPFH is a group of heterogeneous disorders in which there is an increased γ-chain production into adult life (Steinberg, 2001). HPFH can be divided into two types based on the genetic basis. The first type results from a deletion of a variable length in the β-gene cluster. The second type is non-deletional where increased expression of γ-gene results from single point

mutations in the gamma promoter region. In deletional variants, both $^{G}\gamma$ and $^{A}\gamma$ are synthesised in increased quantities. Non-deletional HPFH is associated with mutations in the promoter region of the $^{G}\gamma$- and $^{A}\gamma$-globin genes (Huisman, 1997) resulting in increased expression of gamma genes. In such non-deletion variants, typically only one of the two γ-chains is over expressed.

Distribution of HbF among red cells in HPFH is recognised as either pancellular or heterocellular forms. In the pancellular form, almost every cell contains HbF, whereas in the heterocellular form, only a sub-population of red cells contains HbF. Most HPFH individuals are pancellular forms. The Swiss type HPFH is a heterocellular form which elevates HbF levels to 3.5-6.3% and produces about 10% F^{+}cells or more (Soummer *et al.*, 1981).

Homozygous HPFH is typically asymptomatic, with no clinical finding and no abnormal growth pattern. Such individuals produce 100% HbF with both $^{G}\gamma$ and $^{A}\gamma$ and a slight erythrocytosis (RBC about 6-7 $x10^{6}/\mu l$) due to the higher oxygen affinity of HbF relative to HbA. Their red cells are microcytic with MCV about 75 fl and slightly hypochromic with MCH about 25 pg. Reticulocyte count and bilirubin levels are normal.

Heterozygous HPFH is also asymptomatic with increases in HbF levels from 10-30% of the total Hb. HbA_2 is decreased to 1-2% and the rest is HbA. There is no haematological abnormality and usually found by family study.

δβ-thalassaemia is a form of thalassaemia in which β- and δ-globin chain production is absent, due to deletions and nondeletions of the structural β- and δ-gene complex similar to deletional forms of HPFH. This thalassaemia produces nearly 100% F^{+}cells in homozygous state. However, the homozygous state is very rare. In HPFH, the increased γ-chain production fails to fully compensate for the loss of β-chain production. Thus most patients with δβ-thalassaemia have mild anaemia (Hb levels 10-12 g/dl), hypochromic microcytosis, slight hepatosplenomegaly and some bone change associated with chronic erythroid hyperplasia. Reticulocyte count and bilirubin levels are elevated suggesting some degree of haemolysis. In the heterozygous δβ-thalassaemia, there is no specific clinical finding, no anaemia, and no splenomegaly and such individuals

usually have lower levels of HbF at about 5-20% with usually less than 90% of HbA. The blood picture is similar to that of a heterozygous β-thalassaemia with microcytic, slight hypochromic red cells. HbA_2 is normal or slightly decreased and HbA is usually less than 90% of total Hb. (McKenzie, 1996)

Other factors affecting foetal haemoglobin levels

XmnI polymorphism. This results from a C→T substitution at position -158 of the $^{G}\gamma$-gene promoter which creates an *XmnI* restriction enzyme site associated with an increase in the $^{G}\gamma/^{G}\gamma+^{A}\gamma$ ratio and an increase in HbF levels in both β-thalassaemic and sickle cell patients who carry this mutation (el-Hazmi, 1989; Ballas *et al.*, 1991). Increased HbF levels have also been observed in healthy individuals lacking genes for sickle or thalassaemia disorders who are heterozygous or homozygous for the *XmnI* site (Sampietro *et al.*, 1992).

A region of about 0.5 kb, 1.65-1.15 kb 5' to the $^{G}\gamma$ gene, has been proposed as another area that potentially has a regulatory role in γ-globin gene expression (Pissard & Beuzard, 1994). Designated as the pre-$^{G}\gamma$ framework, this region has several transcription factor binding sites and four polymorphic variants linked to the β-gene cluster haplotype. Whether the pre-$^{G}\gamma$ framework is an important haplotype-linked regulatory region awaits for further study.

Trans-acting elements is another factor that play a major role in the modulation of γ-globin gene expression. Among potential *trans*-acting elements suspected of influencing γ-globin gene expression, an X-linked F cell production (FCP) locus between DXS143 and DXS16 within Xp22.3-22.2 has been identified by linkage analysis but no gene has yet been identified (Dover *et al.*, 1992). This locus may account, in part, for the high HbF levels in females compared to males found in both normal individuals and sickle cell patients. The phenotype of the FCP locus does not influence the HbF response in patients treated with hydroxyurea (Steinberg *et al.*, 1997). HbF production may also be influenced by genetic loci localised on chromosome 6q and by other loci unlinked to the β-globin gene cluster (Craig *et al.*, 1996).

1.7.3. Evidence for protective effect of HbF in sickle cell disorders

New-born protection

In new-born babies with sickle cell disease the sickle forms of red cells are seen less than in adult patients. In 226 pairs of black mothers with sickle cell disease and their affected infants, only about 11% of SS new-born babies showed sickle form of red cells compared to nearly 100% in their mothers blood, (Watson *et al.*, 1948). This finding suggests that high HbF levels in new-born babies have a protective effect against sickling. This is consistent with the absence of clinical episodes of sickle cell disease during the first 4 months of life. Furthermore as the HbF levels decline close to adult levels at about 4 months after birth, the sickle red cells increase gradually, because low levels of HbF no longer give any protection.

Sickle cell disease and hereditary persistence of foetal haemoglobin (HPFH)

Sickle cell patients who are also heterozygous for HPFH produce about 25-30% HbF of the total Hb, and show reduce anaemia and clinical complications despite the presence of 70% HbS (Conley et al., 1963). These observations suggest a protective effect of HbF on the sickling process (Tuan *et al.*, 1980).

Sickle cell patients in eastern oases of Saudi Arabia have unusually high levels of HbF at about 10-26% which are associated with a relatively mild disease (Brown *et al.*, 1972; Perrine *et al.*, 1972). Saudi patients do have painful crises, meningitis and osteomyelitis but less seriously and less frequently. Childhood mortality is lower and there is less anaemia than the other groups of patients. Similar features are also found in sickle cell patients in Southern India (Brittenham *et al.*, 1979).

In sickle cell anaemia, *cis*-acting elements play a role in the modulation of HbF production. Their nature and modes of action are not clearly understood. *Trans*-acting elements also play a role in controlling HbF production in sickle cell disease. The X-linked F cell production (FCP) locus at Xp22.3-22.2 may account,

in part, for the higher HbF levels in the female relative to the male patients. This observation is also found in normal population (Miyoshi, *et al.*, 1988).

Protective effect of HbF induced by hydroxyurea in sickle cell patients

The higher HbF and F^+cell production following hydroxyurea treatment can reduce severity of the disease clearly. This has been mentioned before in section 1.6.4 about the role of HU in SS management.

1.8. OTHER MARKERS IN SICKLE CELL DISORDERS

1.8.1. β-Globin gene cluster haplotypes

Restriction fragment length polymorphisms (RFLPs) can be used as genetic markers to characterise individual genetic differences. RFLPs linked to the β-globin gene cluster define haplotypes of the sickle cell genotypes, which have been named after the geographic locations with the highest prevalence (Nagel & Fabry, 1984) (Table 1.8.1). The β-globin gene haplotype among *cis*-acting determinants serves as one marker for the genetic background inherited with the HbS gene and for elements that may influence γ-globin gene expression (Nagel & Fabry, 1984). There are five haplotypes or genetic backgrounds, Benin, Bantu, Senegal, Cameroon and Arab-Indian, which arose with sickle cell mutation (Pagnier *et al.*, 1984). Diverse genetic determinants inherited along with the β^S gene may indicate possible causes of phenotypic variation in sickle cell disease. The haplotype bearing the β^S gene is associated with a variety of haematological and clinical features that are likely, at least in part, to be mediated through effects on HbF concentration (Nagel & Fleming, 1992). Most of the RFLP sites used to define a haplotype have no known role in the differential transcription and temporal regulation of globin genes. An exception is the *Xmn*I site (-158 C→T) that is 5' to the $^G\gamma$ gene in the Senegal and Arab-Indian haplotypes which is strongly associated with high expression of the $^G\gamma$ gene compared with the $^A\gamma$ gene (Nagel & Fabry, 1984). The Senegal haplotype may

provide the genetic environment necessary for high levels of γ-globin gene expression in the presence of erythroid hyperplasia (Ballas *et al.*, 1991).

Table 1.8.1 Haplotypes associated with the sickle cell mutation

(from Rodgers, 1998)

Haplotype	Frequency[1] (%)	Clinical Features	Hb (g/dl)	HbF (%)
Benin[2]	50-60	severe	8.0-8.5	6-7
Bantu (CAR)[3]	25	most severe	8.0-8.5	6-7
Senegal[4]	15	mild	8.5-9.0	8-9
Cameroon[5]	5	mild-severe	8.0	5-6
Arab-Indian[6]	0	mildest	10.0	15-20
Atypical[7]	18	variable	8.0	5-6

[1]Percentage in African-American population
[2]Benin homozygote
[3]Bantu homozygote and Benin/Bantu combined heterozygote
[4]Senegal homozygote and Senegal/Benin combined heterozygote
[5]Cameroon heterozygote with any typical haplotype
[6]Arab-Indian homozygote
[7]Atypical haplotype heterozygote with any typical haplotype

The Arab-Indian haplotype accounts for more than 90% of the β^S-gene-associated haplotypes in India and is prevalent in the eastern oasis of Saudi Arabia. These patients have high HbF levels and less severe anaemia but have similar vaso-occlusive complications to their compatriots from the western area where the β^S-gene is present on the Benin haplotype (Padmos *et al.*, 1991). In the Saudi Arabian population, the -158 C→T polymorphism is strongly, but imperfectly, associated with high levels of HbF and $^G\gamma$-chain in sickle cell anaemia (Labie *et al.*, 1985). This mutation may play a permissive role, necessary, but insufficient, to promote increased transcription of the $^G\gamma$ gene.

1.8.2. Co-inheritance of α-thalassaemia with sickle cell anaemia

α-Thalassaemia in African origin people usually results from the deletion of one or two α-globin genes on chromosome 16. Nearly a third of Black Africans examined carry at least one α-thalassaemia mutation, so these mutations are frequently found to co-exist with sickle cell mutations (Steinberg, *et al.* 1986). Patients with sickle cell and α-thalassaemia usually have a lower MCV, lower reticulocyte count, less haemolysis and higher haemoglobin concentration compared to with patients with α-thalassaemia alone (Steinberg & Embury, 1986) (Table 1.8.2).

Table 1.8.2 Haematological features of patients with sickle cell and α-thalassaemia (From Steinberg & Embury, 1986)

α-globin genotype	Hb (g/dl)	MCV (fl)	Reticulocytes (%)	HbA_2 (%)
αα/αα	8.0±1.1	92±7	11±6	2.8±0.4
-α/αα	8.6±1.1	83±7	9±6	3.3±0.6
-α/-α	9.2±1.3	72±4	7±5	3.8±0.4

α-Globin genotype shows the number of α-globin genes present in patients with sickle cell anaemia. These data are the averages of values reported in studies of African origin sickle cell patients with α-thalassaemia.

α-Thalassaemia affects the phenotype of sickle cell patients by reducing the red cell haemoglobin concentration. Concurrent α-thalassaemia diminishes the polymerisation potential of HbS in sickle cell anaemia, reducing haemolysis and anaemia. Clinically, the outcome of coincident sickle cell associated with α-thalassaemia is incongruous. Vaso-occlusive events appear undiminished. Fewer dense deformable red cells are found. The coincidence with α-thalassaemia raises the haematocrit. HbF levels are simultaneously increased which would be expected to reduce vaso-occlusion (Phillips *et al.*, 1991). Patients with sickle cell and α-thalassaemia have less painful episodes and less bone complications than those with sickle cell anaemia alone. Nevertheless, more blood transfusions may also be needed to protect against

bone marrow, brain and other organ damages, skin ulcers of the legs and retinal vascular impairments, which are found less commonly in patients with either α-thalassaemia or sickle cell anaemia alone (Rodgers, 1998).

1.8.3. Compound heterozygote characteristics

Clearly the compound heterozygote such as SC and Sβ-thal, may lead to a different phenotype than classical SS. In addition to the classical SS genotype, several compound heterozygous states may also result in an alteration in sickle cell syndromes clinically.

Sickle cell with β-thalassaemia (S/β-thal). Sickle cell with β-thalassaemia has two general genotypes; S/β^0-thalassaemia and S/β^+-thalassaemia. In S/β^0-thalassaemia, the normal β-globin is completely absent. The clinical features appear similar to sickle cell anaemia (SS). Vaso-occlusions are even more common than in SS, probably because these patients have a higher Hb concentration, which raises blood viscosity and slows the blood flow. In S/β^+-thalassaemia, the normal β-globin is present but in reduced levels. The clinical features depend on the type of β^+-thalassaemia mutation. The HbA production varies from very low to high, but HbS always predominates. Patients with a low production of HbA usually present with a similar phenotype to SS or S/β^0-thalassaemia. The majority of S/β^+-thalassaemic patients have about 20-30% HbA with milder clinical symptoms (Kulozik *et al.*, 1991).

Sickle cell with haemoglobin C (SC). Haemoglobin C also results from a single point mutation in the β^6-codon. The codon changes from GAG to AAG which substitutes lysine instead of glutamic acid (β^6 Glu→Lys) (Marotta *et al.*, 1977a; Marotta *et al.*, 1977b). The pathophysiology of SC disease is determined by the specific interaction of HbS with HbC that are present in a near 1:1 ratio in the red cells (Bunn *et al.*, 1982). SC red cells have a decreased cation content but an increased density, which promotes HbS polymerisation. Osmotic swelling of SC red cells reduces their oxygen affinity, the rate of sickling and deoxygenation

induced K^+efflux (Fabry *et al.*, 1982). The SC disease is generally milder and prognosis better than with SS, although some complications are more common. Avascular necrosis of the hip is generally more common in SC than SS. The combined effect of increased haemoglobin level, cell density and blood viscosity may lead to a very high prevalence of retinopathy in SC patients, and is present in about 80% of adult SC patients but uncommon in adult SS patients (Kent *et al.*, 1994).

HbD Punjab or HbD Los Angeles has a substitution of GAG by CAG, glutamic acid to glutamine, at β^{121} (β^{121} Glu→Gln). HbD Punjab is widely distributed among the Sikhs in the Punjab state of India at about 1% of population. It also occurs in the black population in the Caribbean, North America and in Britain especially among Anglo-Indian children. The combination of S and D (SD disease) is typically more severe than SC disease (Serjeant, 1994).

1.8.4. β-Globin gene silencer

A region of DNA of 530 bp 5' to the β-globin gene is an AT-rich region where polymorphic variants are linked to the β-globin gene cluster haplotype. This putative β-globin gene silencer may influence the expression of the β-globin gene by binding a repressor, BP1, whose differential binding may variably inhibit β-globin gene transcription. The evidence is still incomplete but this area could influence the clinical heterogeneity of sickle cell anaemia by suppressing β^S synthesis (Elion *et al.*, 1992).

1.8.5. Haemoglobin A_2 (HbA_2)

HbA_2 the tetramer of 2α- and 2δ-globin chains, inhibits the polymerisation of HbS to the same extent as HbF (Poillon *et al.*, 1993). However it is unlikely that variations in the HbA_2 level affect the phenotype of sickle cell anaemia. When β^0-thalassaemia is caused by a deletion of the 5' portion of the β-globin gene, the HbA_2 level far exceeds than expected. Individuals who inherit this type of mutation with HbS may benefit clinically. Preliminary studies in transgenic mice that over

express the δ-globin gene suggest that too much HbA_2 may injure the red cell, perhaps analogously to HbC, by its strong interaction with the membrane (Nagel, et al. 1995).

1.8.6. Other genetic factors

The effect of genetic differences in vascular function, endothelial cell biology, leukocyte function, platelet function, adhesion molecules on both red cells and endothelial cells, coagulation factors and red cell membrane proteins and lipids on modulation of disease severity are not clearly understood. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is common in sickle cell anaemia. The phenotype of G6PD deficiency with sickle cell anaemia is variable and no conclusion regarding the combination could be drawn (Steinberg *et al.*, 1988). There are many other genetic markers in inherited blood diseases such as red cell membrane content, red cell enzymes, coagulation abnormalities, other haemoglobinopathies and other genetic diseases to be explored that may influence the sickle cell anaemia phenotype.

1.9. ERYTHROCYTE-ENDOTHELIAL INTERACTION IN SICKLE CELL DISORDERS

1.9.1. Adhesion molecules involved in cell-cell interaction

The evidence that the clinical severity of sickle cell disorders may be affected by the degree of sickle red cell adherence to vascular endothelial cells was discussed in section 1.5.4. Sickle red cells adhere to vascular endothelial cells via membrane adhesion molecules (AMs). AMs are expressed on every cell in the body and act as cell to cell or cell to matrix bridges. They may also mediate signals for growth, differentiation, activation, movement and trafficking of cells. In general there are 4 superfamilies of AMs (Moore *et al.*, 1996).

Selectin super-family

Selectins are all transmembrane proteins. They have in common the presence of NH_2-terminal C-type (Ca^{2+}-dependent) lectin-like binding domain, an epidermal growth factor-like region, a variable number of consensus repeat sequences similar to those appearing in complement-regulatory proteins (CRPs), a membrane-spanning region and a short cytoplasmic region. The selectins are important in the early stages of leukocyte adhesion. Stimulation of the endothelium induces leukocytes to roll along the vessel wall. This rolling involves adherence and de-adherence to the endothelium. The weak adhesive force of the selectins is suited for this rolling phenomenon. Activation of the rolling leukocyte by chemokines, cytokines or chemoattractants leads to the shedding of L-selectin from the leukocyte surface. The low density of L-selectin after the shedding allows leukocytes to be localised at inflammatory sites. Selectins can be sub-classified into 3 groups according to the number of CRP domains or their cellular origin (Moore *et al.*, 1996).

L-selectin (leukocyte adhesion molecule 1, LAM-1, CD62L or Lue-8) has 2 CRP domains, a MW of 74-100 kDa, and presents on leukocyte membranes. Its ligands are MadCAM-1, GlyCAM-1 and CD34. Its main functions are bridging among lymphocytes inside lymph nodes and homing of lymphocytes or monocytes to endothelium during adhesion, movement, rolling, flattening and extravasation. The soluble form of L-selectin (sL-selectin) can be detected at increased levels in inflammation and infection, due to its shedding from activated leukocytes.

P-selectin (granule membrane protein-140, GMP-140 or CD62P) has 9 CRP domains, a MW of 140 kDa, and presents on platelets, megakaryocytes and Weibel-Palade body (vWF storage vacuole inside platelet) membranes. It also presents in small amounts on endothelial cells. Its ligand is PSGL-1. Its main functions are bridging between activated platelets and neutrophils or monocytes.

E-selectin (endothelial leukocyte adhesion molecule-1, ELAM-1 or CD62E) has 6 CRP domains, a MW of 95-115 kDa, and presents only on endothelial membranes. Its ligands are ESL-1 and PSGL-1. Its main function is bridging between activated endothelial cells and leukocytes.

Integrin super-family

Integrins are also transmembrane proteins with a C-terminus anchored to the cytoskeleton. They function to receive and transmit external signals into cells and to bridge between the cells and extracellular matrix (ECM). Integrins are non-covalent heterodimers consisting of an α- and a β-chain. At least 14 types of α-chain and 8 types of β-chain have been identified. Integrins are classified into 8 groups according to their β-chain type. There are at least 20 integrins identified so far. Integrins can be recognised by 3 different nomenclatures; their descriptive name, heterodimeric name and cluster designation numeric name (for example, lymphocyte function antigen 1 or LFA-1, $\alpha_L\beta_2$ and CD11a/CD18 are all the same integrin). Integrins can adhere to many types of ECM such as fibronectin, vitronectin, laminin, collagen, vWF, factor X and fibrinogen. The counter-receptors or ligands of integrins in the ECM are oligopeptides, for example, RGD (arginine-glycine-aspartate) in fibronectin, DGEA (aspartate-glycine-glutamine-alanine) and EILDV (glutamine-isoleucine-leucine-aspartate-valine) in other ECMs. Integrins are calcium dependent proteins. (Moore *et al.*, 1996)

β_1-Integrin family (very late antigen or VLA). This family all have a common β_1-chain (VLAβ, gpIIa or CD29) associated with various α-chains. There are at least 9 types of VLA identified so far; VLA-1 ($\alpha_1\beta_1$, CD49a/CD29), VLA-2($\alpha_2\beta_1$, CD49b/CD29), VLA-3 ($\alpha_3\beta_1$, CD49c/CD29), VLA-4 ($\alpha_4\beta_1$, CD49d/CD29), VLA-5 ($\alpha_5\beta_1$, CD49e/CD29), VLA-6 ($\alpha_6\beta_1$, CD49f/CD29), $\alpha_7\beta_1$, $\alpha_8\beta_1$ and $\alpha_V\beta_1$.

β_2-Integrin family (LeuCAM). This family all have a common β_2-chain (CD18) associated with various α-chains. There are at least 3 types of leuCAM identified so far; LFA-1 (lymphocyte function antigen 1, $\alpha_L\beta_2$ or CD11a/CD18), Mac-1 (macrophage 1, $\alpha_M\beta_2$ or CD11b/CD18) and LeuCAMc (complement receptor 4, CR4, $\alpha_X\beta_2$, p150/95 or CD11c/CD18).

β_3-Integrin family (Cytoadhesin). These all have a common β_3-chain (CD61) associated with various α-chains. There are at least 2 types of cytoadhesin identified so far; gpIIb/IIIa ($\alpha_{IIb}\beta_3$, or CD41/CD61) and VNR (Vitronectin receptor, $\alpha_V\beta_3$ or CD51/CD61).

Other β-Integrin families. These have another type of β-integrin associated with various types of α-integrins such as $\alpha_6\beta_4$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_4\beta_7$, $\alpha_E\beta_7$, $\alpha_V\beta_8$.

Immunoglobulin-like super-family

Members of the Ig-like super-family are also transmembrane proteins with a C-terminus anchored to the cytoskeleton and an extracellular N-terminus, which consists of several Ig-like domains. They vary in the number of Ig-like domains. The Ig-like superfamily can be classified into 2 types. C1-types are involved in antigen recognition and include the major histocompatibility (MHC) antigens. C2-types are cellular adhesion proteins and complement ligands and are involved in antigen presentation. These two types are sometimes very difficult to classify due to overlapping functions. There are at least 17 Ig-like members identified so far; ICAM-1 (intracellular adhesion molecule 1 or CD54), ICAM-2 (CD102), ICAM-3 (CD50), VCAM-1 (vascular cell adhesion molecule 1, INCAM-1 or CD106), VCAM-1 alt (alternatively spliced VCAM-1), PECAM-1 (platelet endothelial cell adhesion molecule 1, EndoCAM or CD31), MadCAM-1 (mucosal addressing cell adhesion molecule 1), LFA-2 (lymphocyte function antigen 2 or CD2), LFA-3 (CD58), BL-CAM (B-lymphocyte cellular adhesion molecule or CD22), Cell-CAM-105 (cell-cell adhesion molecule 105), NCAM (natural killer cell adhesion molecule, D2-CAM, NKH-1 or CD56), CTLA-4 (cytotoxic T-lymphocyte adhesin 4), B7 (CD80), B7-2 (CD86), CD4, CD8 and CD48. (Moore *et al.*, 1996)

Miscellaneous

There are many adhesion molecules which cannot be classified into either superfamily such as; sialyl Lewis X, sialyl Lewis A, platelet glycoprotein IV (gpIIIb, thrombospondin receptor, TspR or CD36), platelet glycoprotein Ib/IX

(gplb/IX), H-CAM (CD44), thrombospondin, unusually large vWF, CLA, CR1 (complement receptor 1 or CD35), Endoglin (CD105), Fcε RII (low affinity IgE receptor or CD23), GlyCAM-1 (L-selectin ligand), L-VAP-2, PSGL-1 (P-selectin glycoprotein ligand 1), VAP-1 (vascular adhesion protein 1), CD9, CD34, CD39, CD40, CD40 ligand, CD59 and CD63. (Moore *et al.*, 1996)

1.9.2. Adhesion molecule expression on sickle red cells

CD36 or platelet glycoprotein IV (PltGPIV) or thrombospondin receptor (Tsp.R.) is a 78–88 kDa transmembrane glycoprotein. It is found on megakaryocytes, platelets, endothelial cells, monocytes, reticulocytes and some tumour cell lines. It also presents at increased levels in sickle cell reticulocytes and stress reticulocytes. (Moore *et al.*, 1996)

***CD41/CD61*, CD41** or platelet glycoprotein IIb (PltGPIIb) or αIIb integrin is a transmembrane glycoprotein composed of 2 chains, GPIIbα (120 kDa) and GPIIbβ (23 kDa) linked by a disulphide bond. CD41 is always non-covalently associated with CD61 to form GPIIb/IIIa complex. CD41 is expressed by megakaryocytes, platelets and a small subset of $CD34^{+}$cells (stem cells), suggesting that CD41/CD61 complex is a marker of megakaryocytic lineage. In the resting state the complex binds to immobilised fibrinogen but in the activated stage, it binds to soluble fibrinogen, fibronectin, vWF, vitronectin and thrombospondin. It plays an important role in platelet aggregation. **CD61** or platelet glycoprotein IIIa (PltGPIIIa) or β_3 integrin is a 110 kDa transmembrane glycoprotein normally associated with CD41. CD61 can also associated with CD51 or α_V integrin to form vitronectin receptor (CD51/CD61). **CD41/CD61** is expressed only on megakaryocytes and platelets, whereas CD51/CD61 is found on osteoclasts, endothelial cells, macrophages, fibroblasts, smooth muscle cells, synovial lining cells and renal glomerular cells. CD41/CD61 is also expressed on sickle reticulocytes. It is absent in persons with Glanzmann's thrombasthenia. (Moore *et al.*, 1996)

***CD49d/CD29*, CD49d** or α_4 integrin is a transmembrane glycoprotein of 145kDa without disulphide bonds. CD49d binds non-covalently to CD29 or β_1 integrin to form $\alpha_4\beta_1$ integrin or very late activation antigen 4 (VLA-4) or binds to β_7 integrin to form $\alpha_4\beta_7$ integrin. CD49d in a complex with either β_1 or β_7 integrin is not only involved in cell adhesion to fibronectin and vascular cell adhesion molecule 1 (VCAM-1) but also in intercellular leukocyte interactions. It is expressed on monocytes, lymphocytes, eosinophils, thymocytes and Langerhans cells. It is also expressed on sickle reticulocytes. **CD29** or β_1 integrin or platelet glycoprotein IIa (PltGPIIa) is a transmembrane glycoprotein of 110 kDa that binds non-covalently to α_1-α_8 and α_V integrin. CD29 complexes are involved in cell–cell and cell–matrix adhesion, depending on the types of associated α subunits. They have broad cellular reactivities but are not expressed by erythrocytes. In normal peripheral blood they are found on a subset of CD4+ and CD8+ T lymphocytes and a minority of B-lymphocytes. The inducer subpopulation of CD4+ lymphocytes co-expresses CD4 and CD29. **CD49d/CD29** are also expressed on sickle reticulocytes. (Moore *et al.*, 1996)

1.9.3. Interaction of AM expressing red cells with endothelium

Adhesion molecules (AMs) are expressed on every cell in the body to act as a cell to cell or cell to matrix bridges. They also mediate signals for growth, differentiation, activation, movement and trafficking of cells. Some AMs such as CD36 (glycoprotein IV or GPIV or thrombospondin receptor; Tsp.R.), CD41/CD61(GPIIb/IIIa or αIIb/β3 integrin) and CD49d/CD29 (VLA4 or α4β1 integrin) are expressed on sickle red cells especially in the reticulocyte population and are involved in red cell endothelium interactions. Recent work has demonstrated the importance of red cell membrane AM expression in sickle cell disorders (Moore *et al.*, 1996) by mediating the adhesion of red cells to endothelium. Several AMs which are absent on normal red cells have been found on sickle red cells. In particular, CD36 is known to be expressed on sickle reticulocytes (Hebbel, 1997). Thrombospondin from activated platelets acts as a bridge to adhere sickle red cells to the endothelium either *via* the vitronectin

receptor (VNR) or via CD36 on the endothelial cell surface. CD41/CD61 is also expressed on sickle reticulocytes and binds vWF multimers released from vascular endothelium, thereby acting as a bridge for VNR binding onto the vascular endothelium (Kumar *et al.*, 1996). CD49d/CD29 has been shown to directly link sickle reticulocytes to endothelial VCAM-1 or fibronectin (Gee & Platt, 1995). These mechanisms of adhesion are shown in Figure 1.9.3. Some blood group antigens, such as Lutheran (Le) and LW were also reported to be involved in mediated erythrocyte-endothelial interaction in sickle cell disorders (Parson *et al.*, 1999). Furthermore, *ex vivo* studies have shown that increased expression of AMs on sickle erythrocytes can induce obstruction of blood flow in the microvasculature (Brittain *et al.*, 1993). Adhesion molecules are expressed in increased quantities in reticulocytes (Joneckis *et al.*, 1993) and in a subpopulation of immature reticulocytes with a low density which have been called "stress reticulocytes" (Browne & Hebbel, 1996). Therefore stress reticulocytes carry some adhesion molecules which are normally expressed in the earlier erythroblastic stages in the bone marrow.

Vascular endothelial cells (VECs) may be involved in the sickle vaso-occlusive process as innocent bystanders, since there are many circumstances that alter their native properties. Vascular endothelial cell damage, a trigger for abnormal blood flow, may be due to the damaged membrane of rigid sickle red cells scraping along the side of vasculature, so that oxidant radicals and other vascular endothelial cell activation markers are produced and released into the circulation, exacerbating the crisis (Hebbel *et al.*, 1982). Increased expression of vascular cell adhesion molecules (VCAMs), intercellular adhesion molecules (ICAMs) on VECs and their soluble forms, and other tissue factors have been reported in sickle cell patients. These activated endothelial cells increase in numbers during the vaso-occlusive painful crises (Solovey *et al.*, 1997). The balance of vasodilators and vasoconstrictors may be changed to favour vasoconstriction due to perturbation of the endothelium by the sickle red cell. The vasoconstrictor protein endothelin-1 is increased, while the action of the vasodilator nitric oxide (NO) may be impaired due to abnormal NO production by

the damaged endothelium. Another vasodilator, prostacyclin, may also be low in sickle cell disorders for a similar reason. However, prostacyclin release is subject to adherence of sickle red cells to the endothelial sub-stratum, which occurs only after serious endothelial damage (Sowemimo-Coker *et al.*, 1992).

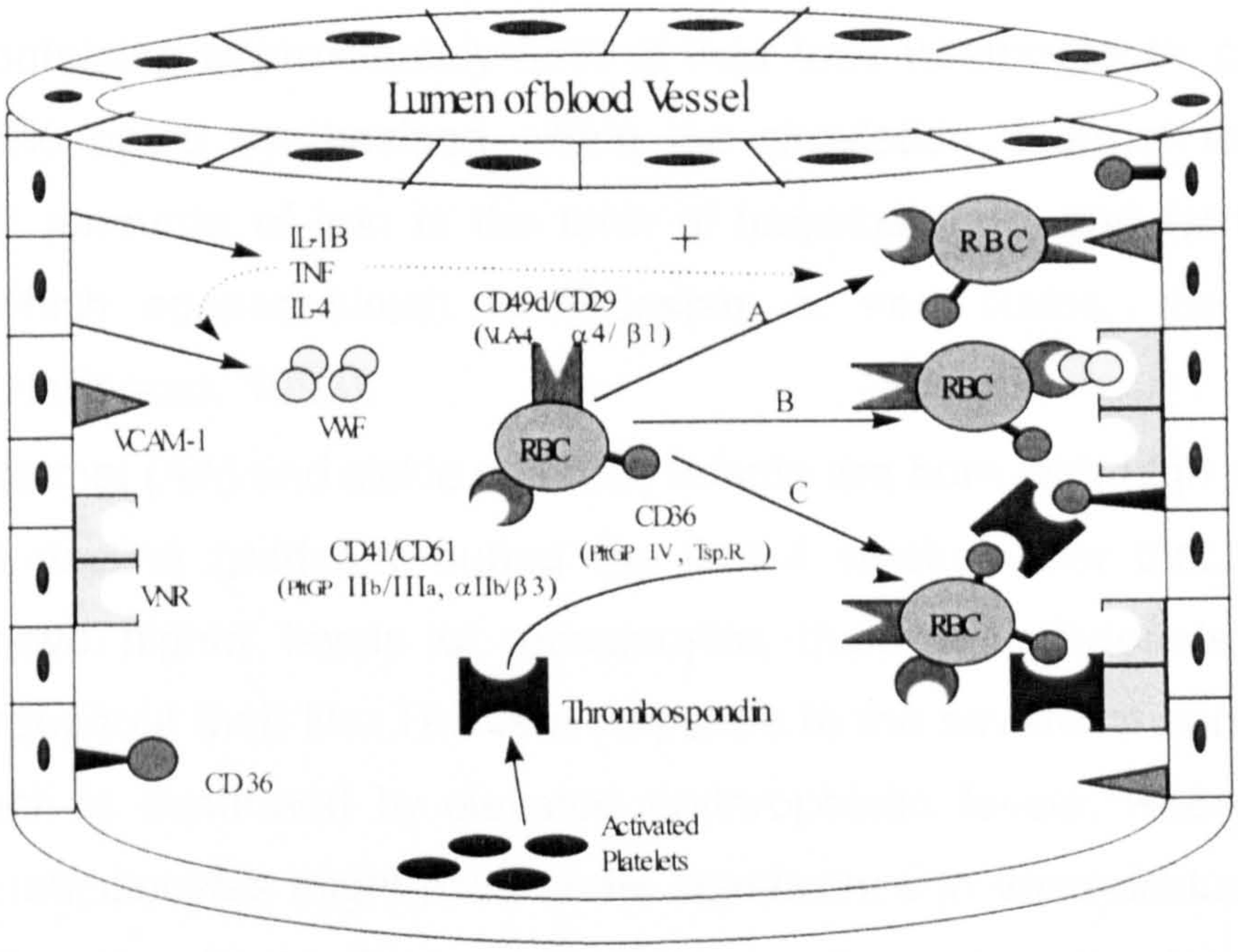


Figure 1.9.3 Erythrocyte-endothelial interactions in sickle cell disorders

(From Moore, et al. 1996)

(A) VLA-4 expressed on sickle reticulocytes adheres directly to VCAM-1 expressed on vascular endothelial cells. VCAM-1 expression is induced by IL-1, TNFα and IL-4.

(B) GPIIb/IIIa expressed on sickle reticulocytes binds to unusually large vWF multimers released from vascular endothelial cells. The reticulocyte-GPIIb/IIIa-vWF complex is bound to vascular endothelial cells via the endothelial vitronectin receptor (VNR).

(C) CD36 expressed on sickle reticulocytes binds to thrombospondin (Tsp). The reticulocyte-thrombospondin complex is then able to bind to vascular endothelial cells via two receptors (VNR or endothelial CD36).

Note: VLA-4 and GPIIb/IIIa are also able to bind to thrombospondin.

1.9.4. Reticulocytes

A reticulocyte is defined as a young red cell with residual RNA and mitochondria but without any nucleus. It stains a bluish colour in routine Wright's stain, the so called polychromatophilic red cell, and shows a bluish reticular appearance in supravital stain (new methylene blue or brilliant cresyl blue), the so called reticulocyte. Reticulocytes are released from the bone marrow into the circulation containing approximately 65% of their total haemoglobin content, the remaining 35% being synthesised within the circulation. Normal reticulocytes contain small amounts of iron in the form of haemosiderin and ferritin in their cytoplasm, which appear bluish in Prussian or iron stains, the so called siderocytes (Hoffbrand, 1993).

Both normal (AA) and sickle cell (SS) infants are born with high reticulocyte levels, which decline gradually during the first 4 weeks. After that, sickle cell individuals have higher levels of reticulocytes than AA individuals, which is sustained throughout their life. This is a response to the severe anaemia in such patients, which is confirmed by elevated erythropoietin levels. Recent findings indicate that reticulocytes might have some significance in vaso-occlusive painful crises in sickle cell patients. They are likely to express more adhesion molecules than mature red cells, and they are also appear to be the first cell to adhere to the vascular wall, before the rigid irreversibly sickle cells are trapped. Reticulocyte levels in sickle cell patients are significantly higher than normal AA persons. (Moore *et al.*, 1996)

1.9.5. Contributions of other adhesive cells to the sickling process

Mature sickle red cells sometimes circulate sticking to leukocytes, platelets or stress reticulocytes. Stress reticulocytes express increased levels of red cell membrane adhesion molecules (AMs) which can bridge to their specific ligands on endothelium to provoke an occlusive process (Sugihara *et al.*, 1992); (Brittain *et al.*, 1993); (Wick *et al.*, 1993) & (Gee & Platt, 1995). Sickle cell patients have increased levels of stress reticulocytes along with other haemolytic anaemia patients, but why the other patients do not present with vaso-occlusion

has yet to be explored. In sickle cell and sickle cell with HbC diseases, hydroxyurea therapy has the capacity to decrease levels of reticulocytes and stress reticulocytes due to its suppression of haemopoiesis, and this reduction appears to correlate well with fewer pain crises (Charache *et al.*, 1996). The influence of neutrophils and possibly eosinophils and basophils, in vaso-occlusion has been increasingly recognised. A high mortality rate correlates with a high granulocyte count in sickle cell disorders (Platt *et al.*, 1994). The greatest reduction in granulocyte and monocyte counts appears to have greatest effect in reducing vaso-occlusive painful crisis episodes in sickle cell patients undergoing treatment with hydroxyurea (Charache *et al.*, 1996). The vascular endothelial cells in sickle cell patients interact with activated neutrophils, which are usually increased in sickle cell patients, then the activated or damaged endothelial cells release a large quantity of many different cytokines and their activation markers into the circulation which exacerbates the painful crisis (Hofstra *et al.*, 1996). Increased platelet counts and increased numbers of activated platelets are found in sickle cell diseases, along with reversible and irreversible changes in the shape of the platelets. Thrombospondin (TSP) is released from activated platelets and can act as a bridge between stress sickle reticulocytes and the endothelium, initiating vaso-occlusion (Brittain *et al.*, 1993).

1.10. ENDOTHELIAL FACTORS AND PLATELET FACTORS

1.10.1. Regulation of VEGF on endothelial cells

Vascular endothelial growth factor (VEGF, also known as vascular permeability factor or vasculotropin) is a hypoxia inducible homodimeric glycoprotein of 35-45 kDa, with specific effects on vascular endothelial cells (VECs) including potent angiogenic, mitogenic and permeability-enhancing effects. VEGF was originally identified as a secreted product of tumour cells (Senger *et al.*, 1983; Senger *et al.*, 1986) and is related to a family of cytokines including placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E

(Maglione *et al.*, 1991; Paavonen *et al.*, 1996). Five isoforms of VEGF have been identified so far, comprising 121, 145, 165, 189 and 206 amino acids, which are generated by alternative splicing from a single gene (Burchardt *et al.*, 1999; Gluzman-Poltorak *et al.*, 2000). $VEGF_{121}$, $VEGF_{145}$, and $VEGF_{165}$ are secreted in soluble form, while $VEGF_{189}$ and $VEGF_{206}$ are cell membrane or extracellular-matrix bound unless proteolytically cleaved (Houck *et al.*, 1992). VEGF mRNA is found in various cell types such as macrophages/monocytes, granulocytes, lymphocytes, megakaryocytes, platelets, mesangial cells, keratinocytes, hepatocytes and endothelial cells (Ferrara *et al.*, 1992). Two high affinity receptors for VEGF found on endothelial cells are the *fms*-like tyrosine kinase receptor 1 (Flt-1) and the tyrosine kinase insert domain-containing receptor (KDR/Flk-1) (Millauer *et al.*, 1993). These two receptors belong to the class III receptor-type tyrosine kinase family and have a high degree of amino acid sequence homology. However, VEGF receptors are now known to be located in uterine smooth muscle (Brown *et al.*, 1997) and pancreatic ductal epithelium cells (Rooman *et al.*, 1997) whereas only Flt-1 is found on monocytes (Clauss *et al.*, 1996). This indicates that VEGF might have some effects on those cells apart from its known effect on VECs.

VEGF is upregulated in response to hypoxia, which is the most specific signal that induces transcription of VEGF genes and stabilises their mRNA (Stein *et al.*, 1995). Hypoxia not only upregulates synthesis and secretion of VEGF, but also modulates its effect *via* the paracrine induction of VEGF receptors in vascular endothelial cells (Flt-1 and KDR/Flk-1) (Viglietto *et al.*, 1996). Whereas hypoxic exposure for 2 hours causes a demonstrable increase in VEGF mRNA in rat lung tissue *ex vivo*, but reaction to such treatment *in vivo* is slower, taking over 28 days for the mRNA to reach a detectable level (Tuder et al, 1995). Hypoxia is also known to increase the expression of a number of other genes encoding vascular cell mitogens produced by endothelial cells such as platelet-derived growth factor B (PDGF-B) and endothelin-1 (ET-1). A 28-bp enhancer in the 5' upstream region of the VEGF gene mediates the expression of VEGF by endothelial cells under conditions of hypoxia (Kourembanas *et al.*, 1997). The

response to hypoxia of the VEGF gene is controlled by two regulatory elements: the first one may be related to the erythropoietin enhancer and the second one appears to be a completely unrelated sequence (Minchenko *et al.*, 1994). The hypoxia-inducible genes for erythropoietin (Epo), tyrosine hydroxylase (TH), and vascular endothelial growth factor (VEGF) are also regulated post-transcriptionally by proteins binding to specific regions located in the 3' untranslated region (UTR) of their mRNAs (Scandurro & Beckman, 1998). Several similarities between the oxygen-sensing mechanisms regulating the expression of VEGF and Epo have been reported, for example expression of both mRNAs is significantly upregulated by hypoxia and cobalt chloride ($CoCl_2$) (Liu *et al.*, 1999), the half-life of both mRNAs is markedly prolonged by cycloheximide (Steinbrech *et al.*, 2000). Hypoxic induction of both Epo and VEGF is also inhibited by carbon monoxide (Goldberg & Schneider, 1994).

Several effects follow VEGF interaction with its receptor on endothelial cells including increased intracellular calcium ion concentration [Ca^{2+}], release of von Willebrand factor (vWF), increased permeability to macromolecules, elongation of endothelial cells and stimulation of endothelial replication (Brock *et al.*, 1991). The earliest effect is an increase of up to fourfold in cytoplasmic calcium concentration [Ca^{2+}]. This effect occurs on cultured endothelium cells (EC) within seconds after exposure to VEGF at low concentrations (Brock *et al.*, 1991). The effect of VEGF on releasing of vWF and other storage substances from EC is similar to that of other EC agonists such as thrombin and histamine. However VEGF appears to act through a different receptor from those agonists, since inhibitors of thrombin and histamine fail to inhibit this action of VEGF (Brock *et al.*, 1991). VEGF was originally known as vascular permeability factor (VPF) according to its potent effect on increasing permeability of microvessels (Brock *et al.*, 1991). Vesicular-vacuolar organelles (VVO), grape-like clusters of uncoated vesicles and vacuoles found at intervals in the cytoplasm of ECs lining venules and small veins, are activated by VEGF. After activation, VVO open their fenestra to allow macromolecules across the cytoplasm from the vascular lumen into the abluminal basal lamina (Kohn *et al.*, 1992). Thus VVOs provide a

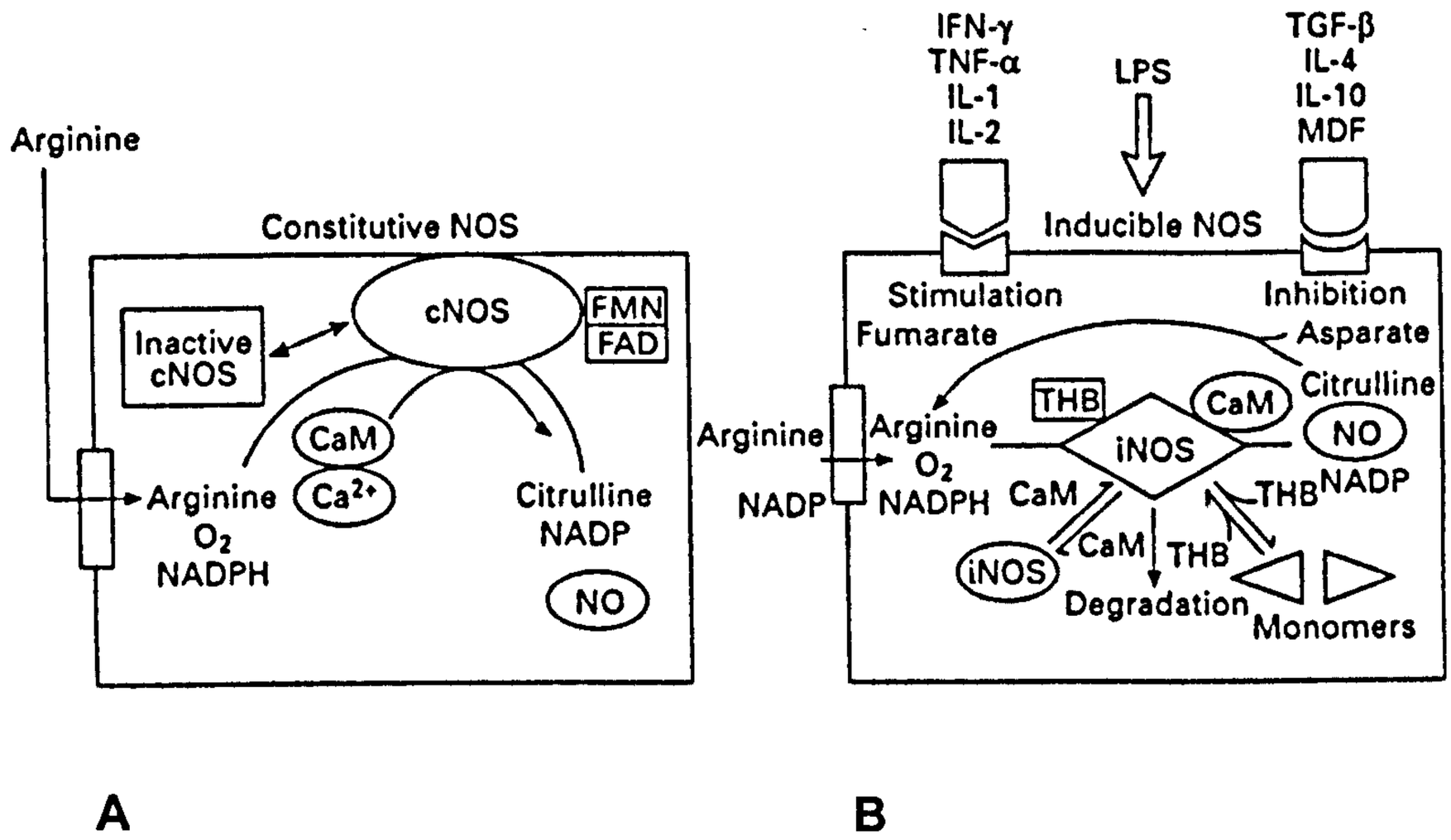
pathway whereby plasma proteins may exit the circulation and enter the tissues (Qu *et al.*, 1995). In addition, VEGF is able to stimulate EC replication, elongation of their shapes and migration to perform angiogenesis (Dvorak *et al.*, 1995a). VEGF is also able to upregulate plasminogen activator, plasminogen activator inhibitor, collagenase, and tissue factors (Dvorak *et al.*, 1995b). This indicates that VEGF is involved in almost all EC functions.

VEGF levels were not documented in sickle cell anaemia (SS) at the time that work on this thesis began. In 1999 there was a report of elevated EDTA plasma VEGF levels in sickle cell patients (120±81 pg/ml) in comparison with healthy blood donors (38±35 pg/ml) (Solovey *et al.*, 1999). That report suggested that the high VEGF levels had an anti-apoptotic effect on circulating endothelial cells (CECs) in sickle cell patients, since CECs in healthy donors showed higher levels of apoptosis than those in sickle cell patients and the apoptosis was related inversely to VEGF levels. However, the cause of increased VEGF levels in that report was not investigated.

1.10.2. Regulation of nitric oxide on endothelial cells

Nitric oxide (NO) is a small molecule consisting of 1 nitrogen and 1 oxygen atom and is a free radical with one unpaired electron. It can move freely throughout the vasculature because of the small size (Darley-Usmar *et al.*, 1997; McAndrew *et al.*, 1997). It is an endogenous vasodilator functioning as endothelium-derived relaxing factor (EDRF) (Palmer *et al.*, 1987). NO is formed by conversion of L-arginine and molecular $O_2^{\cdot}$ to L-citrulline by hydroxylation of one of the L-arginine guanidino nitrogens (Ignarro, 1989). This reaction is catalysed by nitric oxide synthase (NOS) and its co-enzymes, which include reduced nicotinamide adenine dinucleotide (NADPH) and tetrahydrobiopterin (THB) (Moncada & Palmer, 1991; Nathan, 1992).

NOS occurs in 2 forms, constitutive (cNOS) and inducible (iNOS) forms (Forstermann *et al.*, 1994). Schemes for NO synthesis by cNOS and iNOS are shown in Figure 1.10.2.1.



<u>Figure 1.10.2.1</u> Nitric Oxide (NO) synthesis

(From Davies *et al.*, 1995) NO and L-citrulline are formed from L-arginine and molecular O_2 by hydroxylation of one of the L-arginine guanidino nitrogens. There are two distinct NO synthase forms (NOS); constitutive (cNOS) (Figure A) and inducible (iNOS) (Figure B). cNOS and iNOS exist as dimeric and tetrameric complexes respectively. Each unit contains two identical subunits with four prosthetic groups; flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (THB) and haem. The cNOS isoform is activated by humoral, chemical and mechanical signals acting on the cell and is calcium (Ca^{2+}) and calmodulin (CaM) dependent. cNOS associates with the cell membrane and when translocation into the cytosol occurs, its activity is inhibited. NO itself exhibits negative feed back inhibition on cNOS activity. The other isoform; iNOS is induced by endotoxin (ETx), gamma-interferon (IFN-γ), interleukin (IL) I, IL-2 and tumour necrotic factor alpha (TNF-α). However it is inhibited by glucocorticoids, β-transforming growth factor (TGF-β), IL-4 and IL10. It is calcium independent but its active form can bind tightly with CaM. NOS activity is regulated by the intracellular concentrations of various substances such as L-arginine, Ca^{2+}, CaM, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and THB and also some external factors, such as macrophage differentiation factor (MDF), lipopolysacharride (LPS) and shear stress.

NO production occurs in a large number of cell types such as neutrophils, macrophages, fibroblasts, hepatocytes, platelets, endothelial cells, vascular smooth muscle cells and neurones (Bredt, 1999). Constitutive cNOS is distributed in neurones and in endothelial cells. While inducible iNOS is distributed in a wider variety of cell types such as macrophages, endothelial cells and smooth muscle cells. cNOS is now divided into two forms, nNOS or ncNOS

or NOS-I (160kD) and eNOS or ecNOS or NOS-III (133kD). Both are calcium dependent but synthesised from the independent genes and structurally distinct. Constitutive nNOS is mainly distributed in cytosol of neurones and functions as a neuronal messenger. While constitutive eNOS is found primarily in the membrane of endothelial cells and platelets and functions as smooth muscle relaxant. Inducible iNOS or NOS-II (130kd) is cytosolic, calcium independent and can produce as high as nanomolar concentrations of NO. While nNOS and eNOS can produce only picomolar concentrations of NO. The function of iNOS is involved mainly in immunocytotoxicity. The mechanism of action of NO on vascular function is shown in Figure 1.10.2.2.

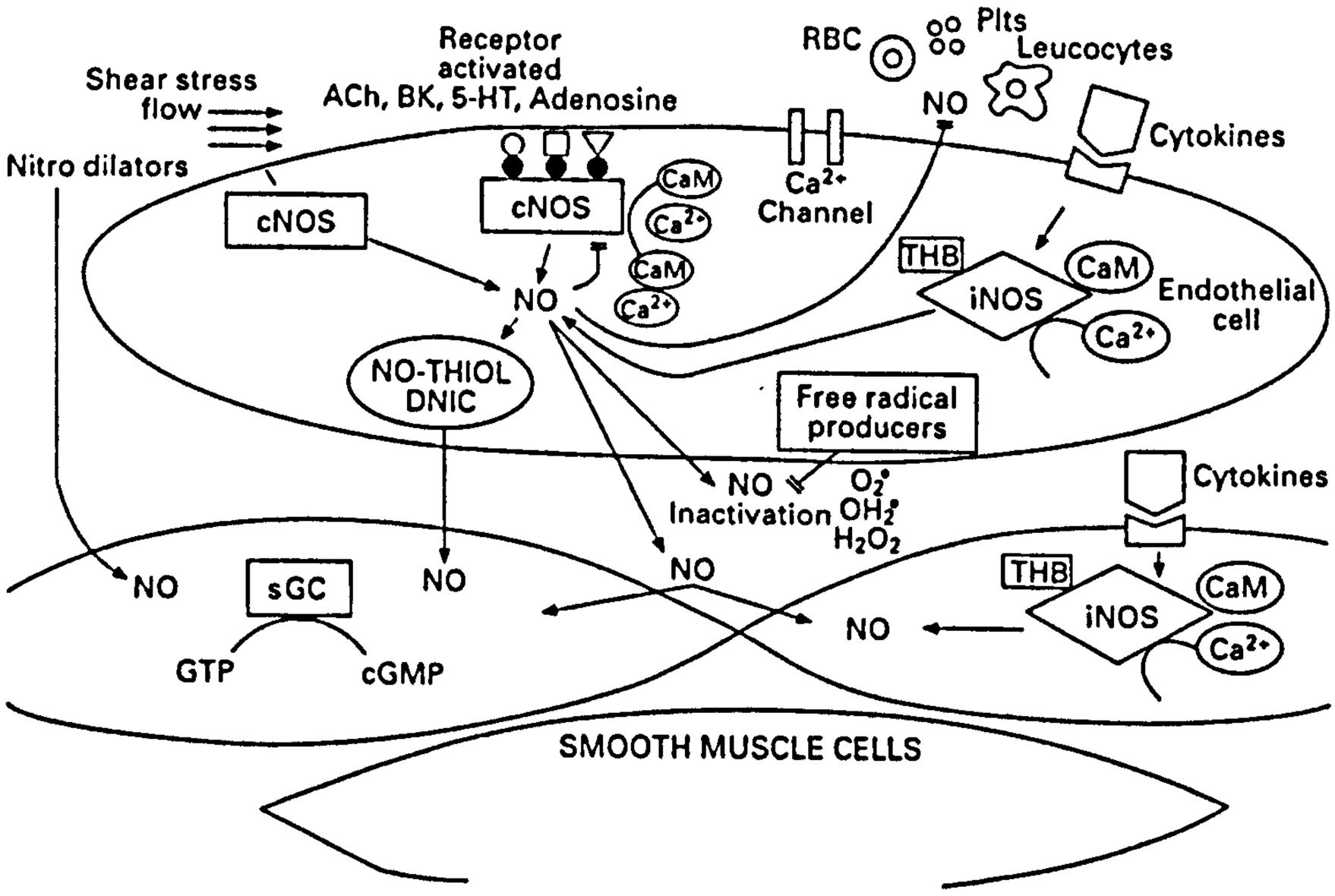


Figure 1.10.2.2 Mechanism of nitric oxide (NO) on vascular function

(From Davies, *et al.* 1995) NO may be formed in the vascular wall by both constitutive nitric oxide synthase (cNOS or ecNOS and ncNOS) and inducible NOS (iNOS). The cNOS is activated by several pharmacological agonists such as acethylcholine (Ach), bradykinin (BK), serotonin (5-hydroxytryptamine, 5-HT) and adenosine, and by mechanical forces such as flow and shear stress. The pharmacological agonists act through G proteins and secondary effectors (indicated by round black dots) to increase intracellular calcium (Ca^{2+}) concentrations. Diffusion of NO from the endothelial cells inhibits the aggregation of red blood cells (RBCs) and platelets (Plts), and adhesion of

leukocytes. NO may be stored intracellularly as nitrosothiol (NO-THIOL) and dinitrosyl iron-cysteine (DNIC). NO acts on soluble guanylate cyclase (sGC) in smooth muscle cells to form guanosine 3',5'-cyclic monophosphate (cGMP) which induces their relaxation. Systemic nitrodilators such as nitrosyl-haemoglobin (NO-Hb) in RBCs supply NO to endothelial and smooth muscle cells. Endothelial cells also produce oxygen free radicals, which can be scavenged or inactivated by NO. In sepsis and during a systemic inflammatory, both endothelial and smooth muscle cells can be induced to upregulate iNOS, which can produce approximately 1000 times more NO than cNOS and is active following induction for up to 20 hr. (CaM = calmodulin, THB = tetrahydrobiopterin, GTP = guanosine 5'-triphosphate)

Increased NO and its metabolites (NOx) have been reported in response to a variety of homeostatic, inflammatory and mitogenic stimuli. In the vasculature, NO acts to resolve ischaemic damage and maintain blood flow (Cristol *et al.*, 1993; Myers *et al.*, 1996). When intracellular Ca^{2+} levels increase, Ca^{2+} binds to calmodulin (CaM; a calcium binding protein) and to cNOS resulting in the stimulation of production and release of NO. iNOS is inducible by cytokines, endotoxin and mechanical injury (Moncada *et al.*, 1991; Xie & Nathan, 1994).

NO is released from endothelial cells in response to growth factors, high intracellular calcium concentration and shear stress (Davies, 1995; Morbidelli *et al.*, 1996). NO has a short half-life of less than 30 seconds in aqueous solution (Ignarro, 1990). This is because it has a high reactivity with adjacent molecules and/or is rapidly oxidised to its final metabolites (NOx), nitrate (NO_3^-) and nitrite (NO_2^-) (Ignarro, 1990). NOx are filtered in kidney and secreted in urine. NO also can react with O_2^- to form $ONOO^-$ (peroxynitrite) a highly cytotoxic species. NO can bind to haem-containing proteins such as haemoglobin (Hb) to form nitrosyl-Hb and circulate within red cells. A similar NO-haem interaction with soluble guanylate cyclase leads to increase intracellular cGMP levels. These stimulate endothelium dependent relaxation of vascular smooth muscle and inhibit platelet aggregation (Schmidt *et al.*, 1993).

Hypoxia inhibits expression of eNOS *via* transcriptional and postranscriptional mechanisms. Hypoxia decreases constitutive NOS (nNOS and eNOS) transcription and protein synthesis in cultured endothelial cells

(Phelan & Faller, 1996). Exposing cultured human umbilical vein endothelial cells (HUVECs) to low pO_2 results in a 40-60% reduction in steady-state eNOS mRNA levels (McQuillan *et al.*, 1994). The lower levels of its mRNA result from a decreased transcription of the gene as well as a reduced mRNA stability. In co-culture experiments, hypoxic HUVECs stimulated significantly less cGMP production in smooth muscle cells and hence presumably less NO synthesis, than the normoxic cells (McQuillan *et al.*, 1994). This inhibitory effect of hypoxia on eNOS production by endothelial cells occurs after 24 h of hypoxia and persists for at least 48 h. (McQuillan *et al.*, 1994). The effects of hypoxia on iNOS appear to be tissue specific. For example, hypoxia upregulates iNOS in a hypoxia inducing factor 1 (HIF-1)-dependent manner in rat pulmonary microvascular but not in aortic smooth muscle cells (Palmer & Johns, 1998). So responses to hypoxia may be different in different tissues. Vaso-occlusion in sickle cell disorders induces local hypoxia and local hypoxia may then be involved in regulating NO production in endothelial cells.

1.10.3. Relevance of NO in sickle cell disorders

Increased plasma NOx levels were reported in sickle cell patients, using Cadmium as a reductant to reduce nitrate to nitrite and then measuring total NOx by the Griess reaction (Rees *et al.*, 1995). This finding is possibly consistent with another report that microvascular endothelial cells in culture exposed to plasma from sickle cell patients with acute chest syndrome can increase NOx levels (Hammerman *et al.*, 1999). Increases occurred within 2 hr of exposure in a concentration-dependent manner and were associated with increases in eNOS protein and its enzymatic activity, but no changes in iNOS transcription, protein nor enzymatic activity were found (Hammerman *et al.*, 1999). The mechanism of this effect in unclear. Endotoxins can upregulate iNOS and endotoxin is increased in some sickle patients in crisis. Since only plasma was used, this could not be a direct effect of hypoxia.

Another report divided sickle cell patients into 3 groups of low, normal and high plasma levels of NOx (Lopez *et al.*, 1996). The high NOx levels were

associated with a low pain score, fewer vaso-occlusion episodes and *vice versa*. This implies that hypoxia-induced ischaemic pain is associated with low levels of NO. Later the same group reported that there was no significant correlation between changes in NOx levels and changes in pain scores in sequential samples taking from early admitted sickle cell patients in emergency department (Lopez *et al*., 2000). These results suggest that low levels of NOx may be an indication of more severe tissue ischaemia.

Other effects of NO in sickle cell disorders may be potentially beneficial or harmful. There is a report that NO inhibits both normal and sickle red cell adherence to endothelium suggesting that enhancement of NO production may have a benefit in sickle cell disease (Space *et al*., 2000). NO also inhibits leukocyte adhesion to the endothelium (Zachary *et al*., 2000). However, a disadvantage of increased NO is reported. NO and/or peroxynitrite ($ONOO^-$), a reactive oxygen species formed from superoxide radical ($O_2^{\cdot}$) and NO, are responsible for initiating cell damage, which leads to apoptosis in kidney cells from transgenic sickle cell mice (Bank *et al*., 2000).

There are some advantages for inhalation of low concentration of NO gas (≤80 ppm) in sickle cell patients during ACS, because of its ability to ameliorate pulmonary hypertension and abnormal ventilation/perfusion. NO may confer some protection against polymerisation of sickle haemoglobin and exert an antiplatelet effect that may be beneficial in ACS (Sullivan *et al*., 1999). NO gas inhalation has been reported to increase the oxygen affinity of sickle cell erythrocytes. Also, a proposed allosteric alteration in haemoglobin, based on S-nitration of the beta-chain at cysteine 93, raise the possibility of altering the pathophysiology of sickle cell disease by inhibiting polymerisation or by increasing NO delivery to the tissues (Gladwin *et al*., 1999a). Augmentation of NO transport to the microvasculature seems a promising strategy for improving microvascular perfusion (Gladwin *et al*., 1999b). There is also some evidence that NO acts as a mediator for hydroxyurea action in clinical improvements in sickle cell patients. Administration of hydroxyurea to rats at clinically relevant doses formed detectable levels of nitrosyl haemoglobin. The NOx detected in

rats resulted from the metabolism of hydroxyurea (Jiang *et al.*, 1997). Nitrosyl haemoglobin has also been detected in hydroxyurea-treated sickle cell patients (Glover *et al.*, 1999). Furthermore, NO is able to inhibit platelet adhesion to vascular endothelium (Radomski *et al.*, 1987). Thus hydroxyurea-derived NO may act as vasodilator and inhibit platelet adhesion to ease clinical symptoms of sickle cell patients.

1.10.4. Relationship of VEGF and NO in the sickling process

VEGF has been shown to have modulating effects on NO, by increasing eNOS. The proliferative effect evoked by VEGF on the cultured microvascular endothelial cells, isolated from coronary postcapillary venules, is reduced by pretreatment with NO synthase inhibitors (Morbidelli *et al.*, 1996). Exposure of the cells to VEGF induces a significant increment in cGMP levels and NO synthase inhibitors abolish this effect. Thus VEGF appears to stimulate the proliferation of postcapillary endothelial cells through the production of NO and cGMP accumulation (Morbidelli *et al.*, 1996). In other experiments (Papapetropoulos *et al.*, 1997), the short-term exposure of HUVECs to 20 ng/ml VEGF for 15 minutes (min) caused significant increments in cGMP levels. This increase was inhibited by N^G-nitro-L-arginine methyl ester (L-NAME, a known inhibitor of NOS) at a concentration of 100 μM. This indicated that the increased cGMP was due to the release of biologically active NO (Papapetropoulos *et al.*, 1997). Thus the short-term exposure, of HUVECs to VEGF, promotes rapid NO release by increasing cytosolic Ca^{2+}, known to be a trigger of eNOS activation. VEGF is also known to increase cytosolic Ca^{2+} concentrations in human endothelial cells (Brock *et al.*, 1991). The longer-term exposure for 24 hours of HUVECs to VEGF has been shown to increase both eNOS protein levels and NO release possibly by the same mechanism (Papapetropoulos *et al.*, 1997). Induction of NO by VEGF was also demonstrated using HUVECs treated with 10 ng/ml VEGF for 1 hr (Hood *et al.*, 1998). In porcine aortic endothelial cells stimulated with VEGF (3, 10, 30 and 100 ng/ml) for 24 hr, an increased expression of both eNOS and iNOS was found only *via* VEGF receptor-2 (KDR/Flk-1), not *via* VEGF receptor-1 (Flt-1) (Kroll &

Waltenberger, 1998). In bovine adrenal cortex epithelial cells (ACE), eNOS expression was induced by VEGF, reaching maximum levels after 2 days of constant VEGF exposure (500 pM), then declining to the base-line levels by day 5 (Shen *et al.*, 1999).

Thus there is good evidence that VEGF induces NO synthesis through the induction of eNOS. How does hypoxia from vaso-occlusion in sickle cell disorders affect NO release in the microvasculature? Hypoxia induces VEGF synthesis taking 24-48 hours (Tuder et al, 1995). Following vaso-occlusion, there will be an induction in VEGF synthesis in endothelial cells of the microvasculature, which by analogy with lung endothelium, may take up to 28 days to reach a maximum (Tuder et al, 1995). This increased VEGF will lead to secondary increased NO release and vasodilatation. However, hypoxia inhibits eNOS rapidly and the effect lasts up to 2 days. Thus vaso-occlusion might be predicted to have a biphasic effect on NO levels, the first being a decrease in NO resulting from eNOS inhibition and the subsequent being an increase in NO through VEGF induced eNOS. A scheme for the effect of hypoxia on VEGF and NO is shown in Figure 1.10.4.

Therefore the work in this thesis examines both NO and VEGF in sickle cell patients in steady state, in crisis and on hydroxyurea treatment, to determine whether vaso-occlusion could result in detectable changes in these markers.

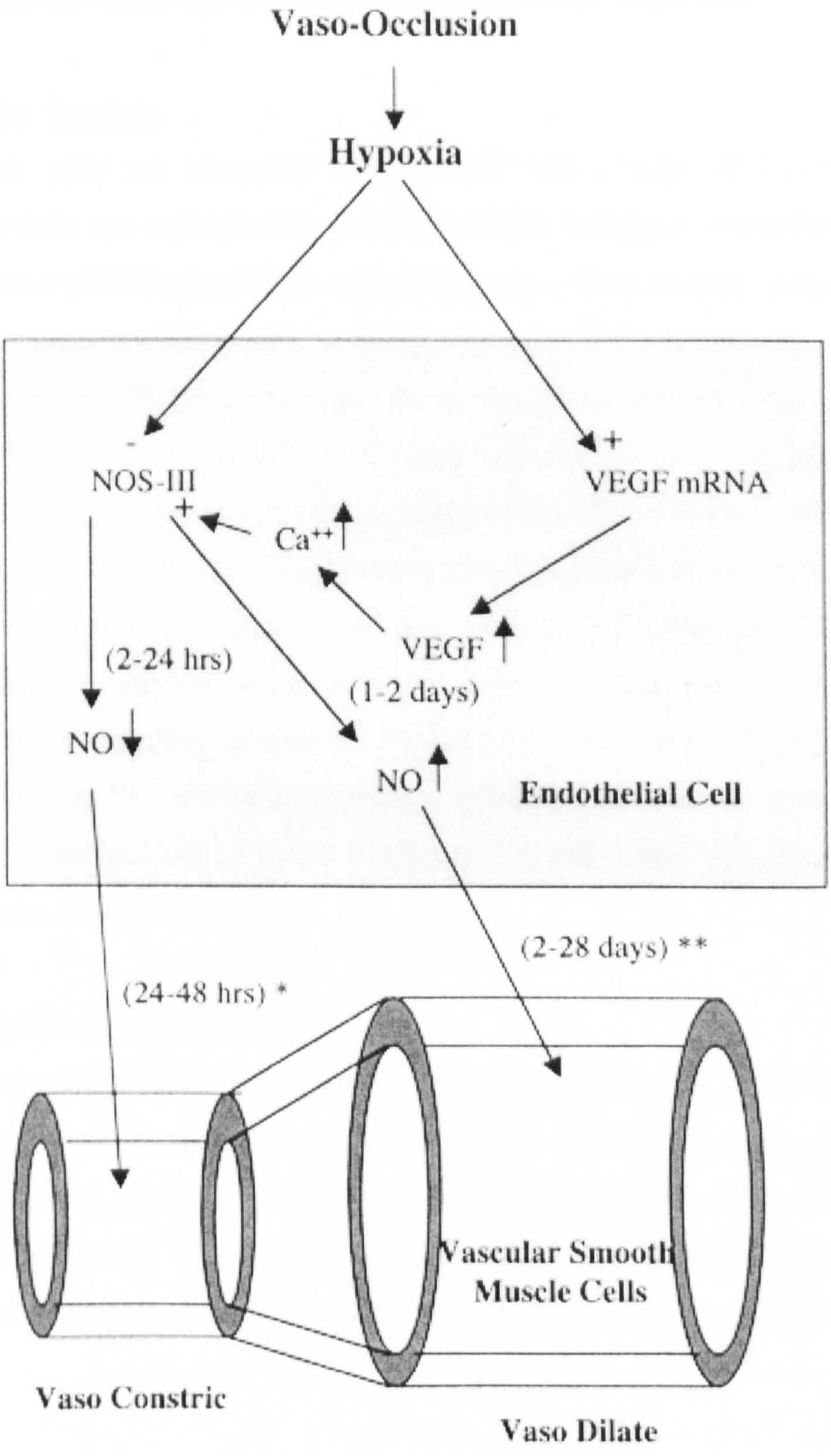


Figure 1.10.4 Effect of hypoxia on VEGF and NO

* From (Papapetropoulos *et al.*, 1997) and ** from (Tuder et al, 1995).

1.10.5. Platelet activation and its relevance in sickle disorders

Normal platelet function

Platelets play an essential role in the first phase of the haemostatic process. Platelets are activated by subendothelial collagen, microfibrils and von Willebrand factor (vWF) after blood vessel damage. This makes platelets adhere to exposed subendothelial matrix and aggregate to form a thrombus plug at the site to stop bleeding. Platelets can also be activated by several agonists such as adenosine diphosphate (ADP), thrombin, epinephrine, 5-hydroxytryptamine, thromboxane A2, prostaglandin H2, prostaglandin G2, platelet-activating factor and vasopressin. After activation, platelets change their shape from the normal discoid to develop dendritic extensions and release the contents of their storage granules. Dense-granules release ADP, calcium and serotonin which can activate nearby circulating platelets. Alpha-granules release BTG, PF4, vWF, fibrinogen, fibronectin, thrombospondin, platelet derived growth factor and plasminogen activator I. All of these molecules can be used as platelet activation markers both *in vivo* and *in vitro*.

Platelet activation in sickle disorders

Evidence of platelet activation *in vivo* in sickle cell patients has been reported using various markers (Browne *et al.*, 1996). However, there is one report of evidence against platelet activation *in vivo* of sickle cell patients (Buchanan & Holtkamp, 1983). Furthermore, VEGF is found to be synthesised in megakaryocytes and stored in platelets (Adams *et al.*, 2000).

Prevention of platelet activation *in vitro*

It is therefore important to determine whether serum or plasma is the most suitable for *in vivo* platelet activation study, and to take any necessary steps to minimise the contribution of platelet activation *in vitro* after blood collection. Therefore in order to avoid *in vitro* platelet activation, a special anticoagulant which can inhibit platelet activation *in vitro* was used instead of conventional anticoagulant. CTAD anticoagulant contains citrate, theophyline, adenosin and dipyridamol, designed for suppression of *in vitro* platelet activation. Theophyline enhances the action of prostaglandin E1 (PGE1) which blocks platelet activation (Hess *et al.*, 1975) and dipyridamol stimulates biosynthesis of prostacyclin, another platelet activation inhibitor (Mentz *et al.*, 1981). Two platelet activation markers: BTG (a homotetrameric protein of 35.8 kDa, produced by platelets and stored in α-granules) and PF4 (a homotetrameric platelet-specific protein of 30kDa, produced by platelet and also stored in α-granules) together with VEGF and NOx were examined in CTAD plasma from sickle cell patients as well as control subjects, looking for any evidence of platelet activation *in vivo*. Since activated platelets may release both VEGF (Banks *et al.*, 1998; Webb *et al.*, 1998) and NOx (Ichiki *et al.*, 1996; Marietta *et al.*, 1997).

1.10.6. Endothelial activation and its relevance to sickle cell disorders

Vascular endothelial cells (VECs), which form the inner lining of blood vessels, play a pivotal role in regulating blood flow. They also actively control tissue homeostasis, vascular tone, blood-tissue exchange, coagulation, fibrinolysis and cellular trafficking. VECs produce and secrete many biological substances into the circulation and/or subendothelial extracellular matrix. These include von Willebrand factor and tissue factors that participate in platelet adhesion and coagulation, prostacyclin, thrombomodulin, and heparan sulphate, which contribute to antithrombotic properties of VECs, plasminogen activator and plasminogen inhibitors, and are essential for controlling fibrinolysis. VECs are the source of molecules regulating their growth and other cell growth. They produce angiotensin-converting enzyme, and act as target cells for lipoprotein

and hormone binding. Finally, they are the target for, and participate in, immune reactions. VECs contribute to the regulation of blood pressure and blood flow by releasing vasodilators such as nitric oxide (NO) and prostaglandin I 2 (PGI_2), as well as vasoconstrictors including endothelin 1 (ET-1) and platelet activating factor (PAF). These compounds are not stored in intracellular granules but their synthesis and release are rapidly activated through specific receptors on VECs (Cines *et al.*, 1998). Thus, endothelial cells constitute not only the first barrier between the blood and the extravascular space but also serve as a source of molecules influencing the structural and functional integrity of the circulation.

VECs also express cell membrane adhesion molecules (CAMs) which act as binding sites for leukocytes and platelets during adhering, rolling and extravasation. E-Selectin (ELAM-1 or CD62E) is the specific CAM which is expressed predominately on post-capillary venules (Bevilacqua *et al.*, 1989). VCAM-1 (INCAM-110 or CD106) is another CAM, which is also expressed on dendritic cells. ICAM-1 (CD54) is more broadly distributed, being expressed not only on VECs but also on monocytes, thymocytes, fibroblasts, chondrocytes and dendritic cells (Gearing *et al.*, 1992). These CAMs are normally expressed at a very low level. They are upregulated rapidly after activation by inflammatory cytokines such as IL-1 and TNF-alpha (Pigott *et al.*, 1992). Alpha4beta1 (VLA4 or CD49d/CD29) (Walsh *et al.*, 1991) and beta2 integrin (CD43) (Rosenstein *et al.*, 1991), are known to be specific ligands for VCAM-1 and ICAM-1 respectively. Sialyl Le (x) and sialyl Le (a) are believed to be E-Selectin ligands (Berg *et al.*, 1992). Soluble forms of CAMs are present in cell culture supernatants (Pigott *et al.*, 1992) and in the serum or plasma of healthy individuals but are significantly increased in some types of malignancies (Harning *et al.*, 1991). There is no evidence for alternatively spliced forms of CAMs, suggesting proteolytic cleavage from the cell surface as a mechanism of production of soluble forms. This mechanism is thought to be a negative feed back loop for limiting adhesion when the inflammatory site gets enough cells to combat the invaders or injuries. Soluble CAMs can also neutralise ligands on activated target cell surfaces thus reducing adhesion. Soluble E-Selectin can also be a chemo-attractant for

neutrophils and can stimulate the adhesive activity of leukocyte integrin CR3 (CD11b/CD18, Mac-1, alpha m beta 2) on human neutrophils (Lo *et al.*, 1991). This indicates that soluble CAMs not only neutralise their ligands but also have some more physiological and pathological significance.

Endothelial cells may be activated directly by hypoxia, which is clearly of potential relevance to vaso-occlusion in sickle disorders. Hypoxia appears to regulate the synthesis and release of vasoactive substances involved in modulating vascular smooth muscle tone and remodelling the vasculature and surrounding tissue (Vane *et al.*, 1990). The Oxygen tension (PO_2) in arterial blood is approximately 150 mm.Hg., in tissues is 40 mm.Hg. whereas around 15-30 mm.Hg. is defined as hypoxaemic conditions. However endothelial cells are able to sense and respond to PO_2 below 70 mm.Hg. (Faller, 1999). A variety of cells including VECs respond to hypoxia by increasing synthesis and release of the vasoconstrictors, ET-1 and PAF and also vasodilators, NO and PGI_2 to control vascular tone and blood flow (Cines *et al.*, 1998). VECs are found to decrease eNOS transcription and its protein levels under hypoxia (Phelan & Faller, 1996) but hypoxia has the opposite effect on iNOS. Hypoxia also induces synthesis and release of erythropoietin (Lacombe *et al.*, 1988) as well as VEGF (Tuder *et al.*, 1995).

In sickle cell patients in steady state (non-symptomatic patients), a high serum level of sVCAM-1 has been observed compared to the controls (Duits *et al.*, 1996). In sickle cell patients in crisis, the levels of sVCAM-1 increase even more and seem to correlate with crisis development (Duits *et al.*, 1996; Stuart & Setty, 1999). Perfusion with sickle erythrocytes in cultured human endothelial cells, can upregulate ICAM-1 and VCAM-1 gene expression (Shiu *et al.*, 2000), suggesting a direct stimulating effect of sickle erythrocytes.

Numbers of circulating endothelial cells (CECs) were significantly higher in the sickle cell patients than in the healthy subjects but the numbers in the crisis SS were not significantly different from steady state SS (Sowemimo-Coker *et al.*, 1989).

Two endothelial cell activation markers: soluble endothelium selectin (sE-Selectin, a transmembrane protein of 115 kDa, specific marker for VEC activation or damage) and soluble vascular cell adhesion molecule 1 (sVCAM-1, another transmembrane protein of 100-110 kDa, a marker for VEC activation and damage but not restricted to VECs) were studied in order to compare the degree of endothelial activation in sickle cell patients and in control subjects.

1.10.7. Other factors modulating red cell endothelial interaction in sickle cell disorders

Inflammatory factors. Tissue necrotic factors, IL-1 and IL-4 are released in response to the inflammatory process associated firstly with vaso-occlusion and then with tissue hypoxia and cell damage. These factors are involved in increasing expression of VCAM-1 (the receptor of VLA-4) and E-selectin on endothelial cells which promote red cell adhesion to endothelial cells (Natarajan *et al.*, 1996)

Von Willebrand factors (unusually high molecular weight). This high molecular weight vWF is released from endothelial cells in response to endothelial activation due to vaso-occlusion. It then mediates the adhesion between gpIIb/IIIa (CD41/CD61) on the red cell membrane to vitronectin receptor (VNR) on endothelial cells. (Wick *et al.*, 1993)

Erythropoietin (Epo). Production of Epo is regulated by changes in oxygen availability. Epo levels increase in response to hypoxia or anaemia. Increased levels of Epo in sickle cell disease are due to the anaemic state of the patient. Epo has no direct modulatory effect on vaso-occlusion but anaemia secondary to red cell damage as a consequence of vaso-occlusion and extravascular haemolysis may play a role as an indirect indicator for the vaso-occlusion. (Roger *et al.*, 1991)

1.11. AIMS OF STUDY

Development of flow cytometric methods for:

Single-colour staining using either FITC or TC for F^+cells assay.

Simplify the procedure above and compare to the original procedure.

Double-colour staining for simultaneous assay of F^+cells, reticulocytes and F^+reticulocytes.

Three double-colour staining for co-distribution of HbF, RNA and AMs.

Triple-colour staining for co-distribution of HbF, RNA and AMs.

Contribution of foetal haemoglobin and red cell adhesion molecules to sickle red cell survival comparing to normal healthy controls

F^+cells, F^+reticulocytes and enrichment ratios.

Adhesion molecule expression on red cells, reticulocytes and F^+cells.

Co-distribution of adhesion molecule and foetal haemoglobin.

Paired data analysis of red cell sub-population in the same patients.

Follow up of a hydroxyurea treated sickle cell patient.

Endothelial activation markers, platelet activation markers and anaemic markers in sickle cell disorders:

Levels of VEGF and NOx in serum and plasma.

Effect of *in vitro* platelet activation on release of VEGF, NOx and others.

Relationship between plasma levels of VEGF and NOx.

Relationship between plasma levels of VEGF, NOx and platelet count.

Relationship between plasma levels of BTG, PF4 and platelet count.

Relationship between plasma levels of VEGF, NOx and platelet activation markers (BTG and PF4).

Relationship between plasma levels of VEGF, NOx and endothelial activation markers (sE-Selectin and sVCAM-1).

Relationship between plasma levels of VEGF, NOx and anaemic markers (Hb and Epo).

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. BLOOD SAMPLES

Normal control blood samples were taken from University College London postgraduate students and laboratory personnel. Sickle blood samples were obtained from sickle cell patients attending University College London Hospitals. Serum and plasma from EDTA, citrate, heparin and CTAD anticoagulated blood samples were stored at –70°C for VEGF, NOx, Epo, sE-Selectin, sVCAM-1, BTG and PF4 assays. Red cell samples from either EDTA, citrate, heparin or CTAD anticoagulated blood were mixed with Alsever: PBS mixture (1:1) to approximately 45-50% haematocrit and stored at 4°C for flow cytometric assays. Haemoglobin typing and quantitation were done by HPLC. Full blood counts (FBC) and reticulocyte counts were run on a blood cell analyser.

2.2. REAGENTS

- Antibodies to mouse globulin conjugated to RPE and to biotin were purchased from Serotec, Oxford, UK.
- Bovine serum albumin (BSA) powder was purchased from Life Technologies, Paisley, UK.
- BTG and PF4 ELISA kits were purchased from Diagnostica Stago, Asnieres-Sur-Seine, France.
- EDTA, citrate, heparin, CTAD anticoagulant and plain vacuum tubes for blood collection were purchased from Becton Dickinson, San Jose, CA, USA.
- Glutaraldehyde was purchased from Sigma, Poole, UK.
- HPLC reagent kits were purchased from BioRad, Hercules, CA, USA.
- Isotype controls: mouse IgM, IgG1 and IgG1 conjugated to FITC and to TC were purchased from CalTag, Burlingame, CA, USA.
- Monoclonal antibodies to human CD36, CD41 and CD49d were purchased from Serotec, Oxford, UK.

- Monoclonal antibody to human foetal haemoglobin conjugated to FITC and to TC was purchased from CalTag, Burlingame, CA, USA.
- Nitrate and Nitrite calorimetric assay kits were purchased from Cayman Chemicals, Ann Arbor, MI, USA.
- Phosphate buffer saline (PBS) tablets were purchased from Oxoid, Basingstoke, UK.
- RPMI, FBS, P/S solutions were purchased from Life Technologies, Paisley, UK.
- Streptavidin conjugated to RPE was purchased from CalTag, Burlingame, CA, USA.
- Thiazole orange (TO) 100 ng/ml in PBS (Retic Count Reagent) was purchased from Becton Dickinson, San Jose, CA, USA.
- Thrombin activating protein (TRAP) was purchased from Sigma, Poole, UK.
- Triton X-100 was purchased from Sigma, Poole, UK.
- VEGF, Epo, sE-selectin and sVCAM-1 ELISA kits were purchased from R&D Systems, Abingdon, UK.

2.3. SOLUTIONS AND MEDIA

- 0.05% Glutaraldehyde in PBS (100 µl 25% glutaraldehyde in 50 ml PBS). It can be stored at 4°C for up to 4 weeks.
- 0.05% Triton X-100 in 0.1% BSA-PBS (25 µl triton X-100 in 50 ml 0.1% BSA-PBS). It can be stored at 4°C for up to 4 weeks.
- 0.1% Triton X-100 in 0.1% BSA-PBS (50 µl triton X-100 in 50 ml 0.1% BSA-PBS). It can be stored at 4°C for up to 4 weeks.
- 0.1% Bovine serum albumin in PBS (0.5 g BSA powder in 500 ml of PBS). It can be stored at 4°C for up to 4 weeks.
- 1% Formaldehyde in PBS (500 µl formaldehyde in 50 ml PBS). It can be stored at 4°C for up to 2 months.
- Alsever's solution (0.33 g chloramphenicol, 0.50 g citric acid, 20.5 g dextrose, 2.0 g inosine, 0.5 g neomycin sulphate, 4.2 g sodium chloride, and 8.0 g tri-sodium citrate dihydrate in 1 litre distilled water). It can be stored at 4°C for up to 2 months.

- Phosphate buffered saline (1 PBS tablet in 100 ml distilled water). It can be stored at RT for up to 4 months.
- 1:1 Alsever's solution: PBS (100 ml Alsever's solution plus 100 ml PBS). It can be stored at 4°C for up to 2 months.
- Reticulocyte culture medium (500 ml RPMI-1640 supplemented with 63 ml citrate phosphate dextrose adenine formula 1; CPDA-1: 327 mg citric acid monohydrate, 251 mg sodium dihydrogen phosphate dihydrate, 2.63 g sodium chloride, 3.19 g dextrose monohydrate and 27.5 mg adenine in 63 ml distilled water; 10% FBS, and 1 IU/ml penicillin plus 1 ng/ml streptomycin (P/S). All ingredients were sterilised by passing through millipore filter before mixing.) It can be stored at 4°C for up to 4 weeks.
- Thrombin activating protein (TRAP) solution (480 μM TRAP in PBS). It can be stored at 4°C for up to 4 weeks.

2.4. EQUIPMENT

- Blood cell analyser model Sysmex SE-9500 (plus reticulocyte count) came from Sysmex, Kobe, Japan.
- ELISA reader model MR-700 came from Dynatech Laboratories, Guernsey, UK.
- Flow cytometer model Coulter Epic XL-MCL came from Coulter-Beckman, Luton, UK.
- HPLC analyser model BioRad VARIANT came from BioRad, Hercules, CA, USA.

2.5. METHODS

2.5.1. Flow cytometry

Principles of flow cytometry

Flow cytometry is the analysis of cellular or particle characteristics in a fluid suspension as they pass singly through a beam of light. The scattered light (both forward or low angle and side or 90° angle) and fluorescent intensity are measured

by a set of photodetectors (photomultiplier-diode tubes). The data collected by these detectors is transferred into a computer for further processing, analysis and display of the results. There are four basic compartments to a flow cytometer; the fluidic system, the optical system, the detecting system and the data processing system.

The function of the fluid system is to create a stream of single particles or cells flowing through the flow cell or flow chamber from the sample tube through to the waste tank. This is achieved by hydrodynamic focusing, in which the sheath fluid is wrapped around the test sample and both are pumped together in the same direction along a tiny tube and then ejected directly through the flow chamber. The sheath fluid acts as a fluid ring singly escorting the sample particles through the flow cell (Figure 2.5.1.1). The flow rate can be adjusted to allow between less than a hundred to over a thousand particles passing through the flow cell per second.

The function of the optical system is to create a light beam, usually a laser beam because of its monochromatic nature and high intensity, to project directly into the flow cell and hit the particles with high intensity. This causes the particles to show their specific characteristics of different forward (low angle) and side (90°) light scatter and fluorescence (only in case that they are stained by fluorochromes).

The function of the detector system is to detect all light and/or fluorescent signals emitted by the specific characteristics of the sample particles after they are hit by the laser beam (Figure 2.5.1.2).

Finally, the function of the data processing system is to integrate the signals and display them in an understandable output such as single channel histogram (the signal from one detector plotted against the number of the detected particles), double-channel or dot-plot histogram (a single particle plotted as a dot in between two axes of signal from two different detectors) and triple-channel or prism histogram (the signal from the combination of three different detectors plotted against the number of the detected particles) (Figure 2.5.1.3).

Flow cytometry is a more powerful analytical tool than light or fluorescent microscopy because of the larger number of cells analysed. The powerful computer allows the possibility of re-analysis using different ways of gating and

cut-off criteria. The limitation of flow cytometry is that the samples have to be free particles suspended in fluid. Therefore, solid tissues have to be digested into single cells before analysis.

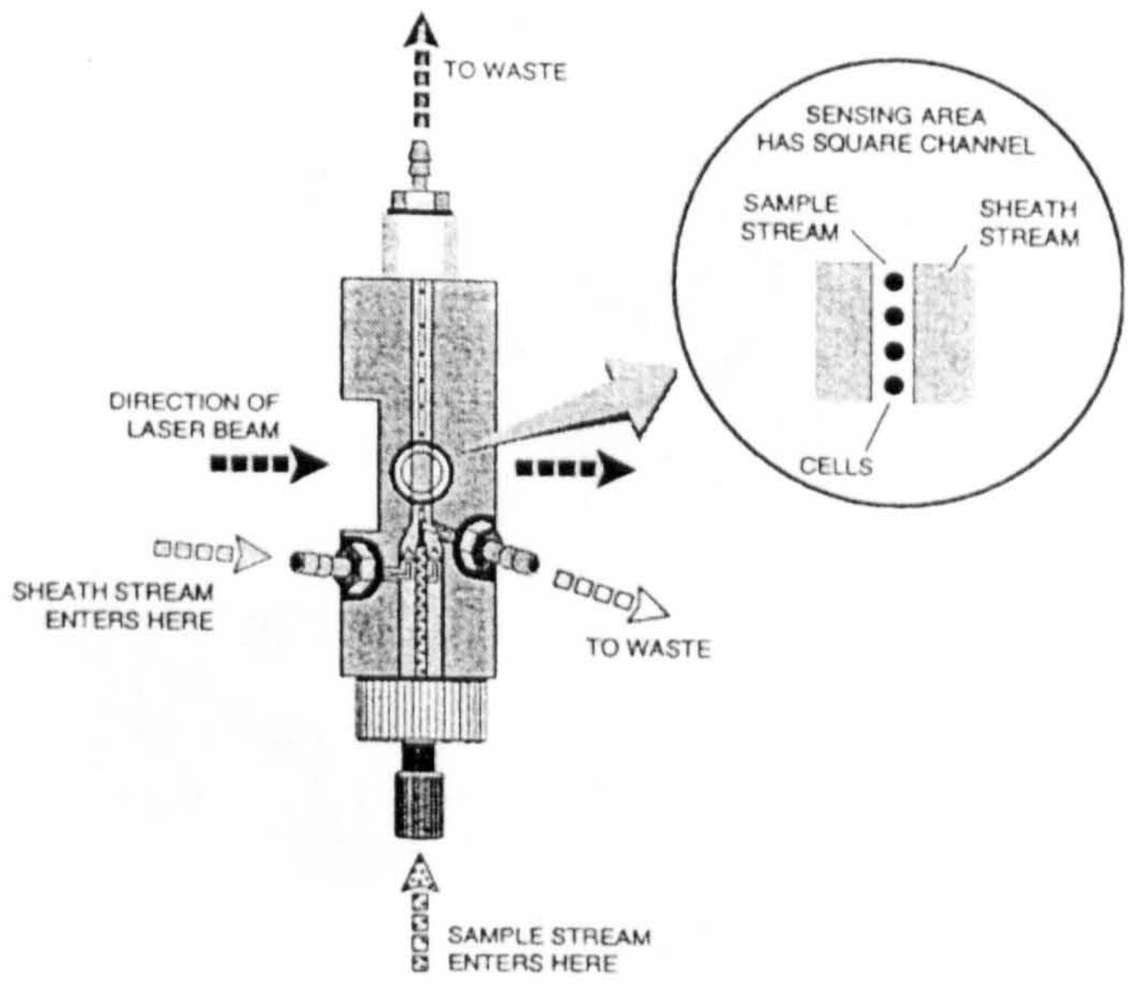


Figure 2.5.1.1 Flow cell of Coulter Epics XL-MCL flow cytometer

(From Coulter Epics XL-MCL instruction manual). The process begins with the flow of isotonic saline solution called the sheath fluid. The sheath fluid enters the flow cell from beneath and flows upward by positive pressure pump. The sample, which contains particles or cells in suspension also, flows upward, using the same pump. A single particle flows directly through the centre of the flow cell because of the pressure of the sheath fluid against it. This is called hydrodynamic focusing. The laser beam is projected through the centre of the flow cell. The sample particles hit by the laser are excited and emit a fluorescent signal that can be detected by the detector.

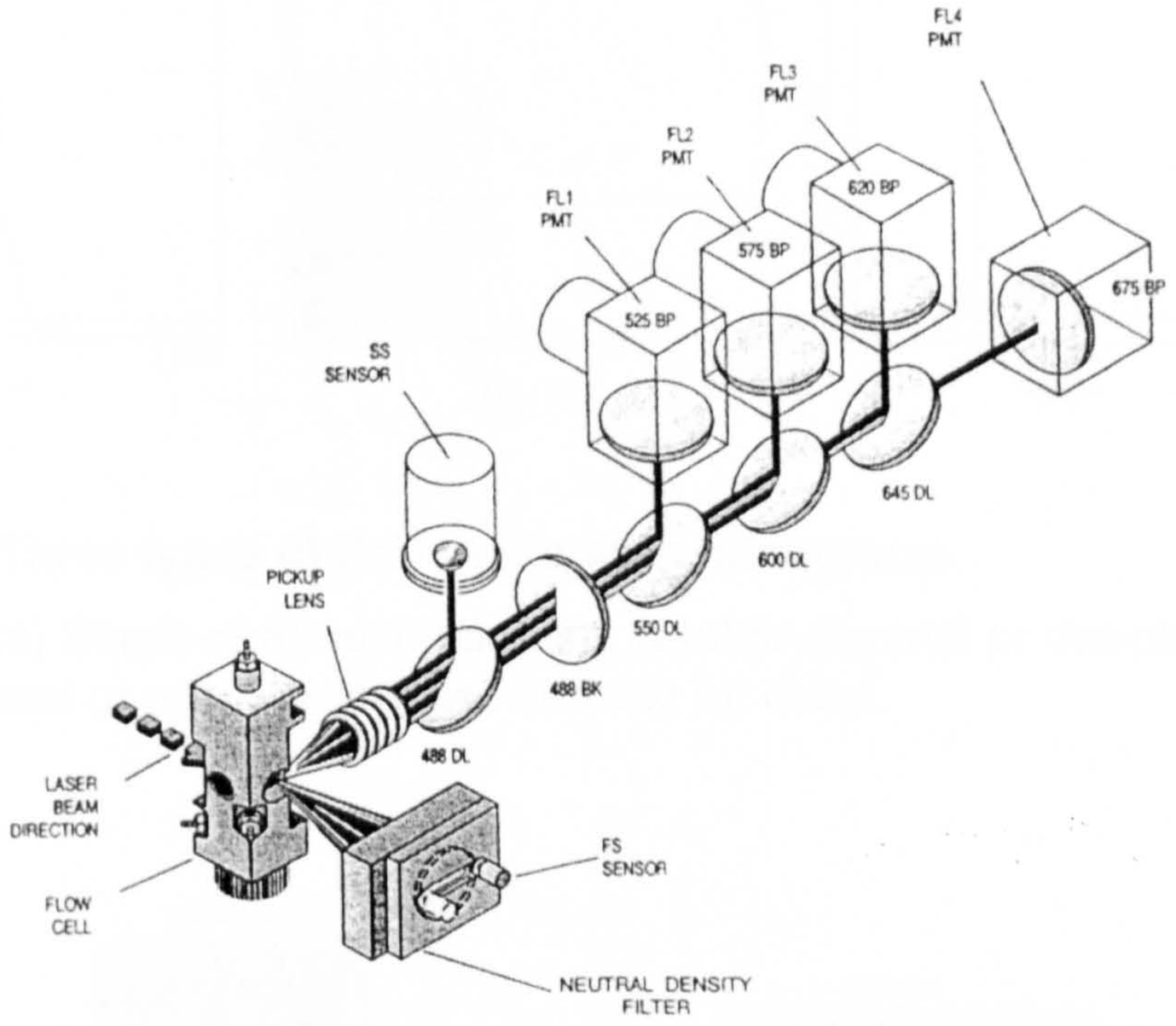


Figure 2.5.1.2 Optical system of Coulter Epics XL-MCL flow cytometer

(From Coulter Epics XL-MCL instruction manual). The optical system includes forward scatter (FS), side scatter (SS), fluorescent channel 1 (FL1 for ~525 nm), FL2 (for ~575 nm), FL3 (for ~620 nm) and FL4 (for ~675 nm) respectively. Each channel has a photo-multiplier tube for signal acquisition. The signals are then transferred to a computer for further processing and display of results.

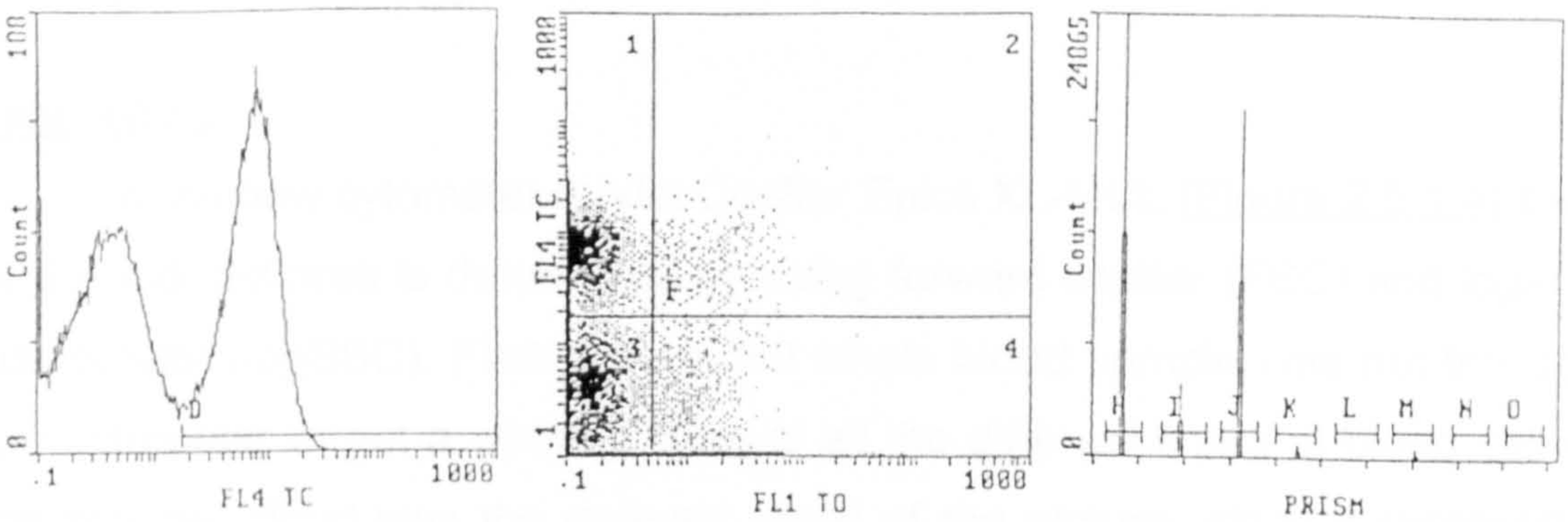


Figure 2.5.1.3 Three types of flow cytometric histograms

(From left→right) Single-channel histogram, Double-channel or dot-plot histogram and Triple-channel or prism histogram. See text for detail.

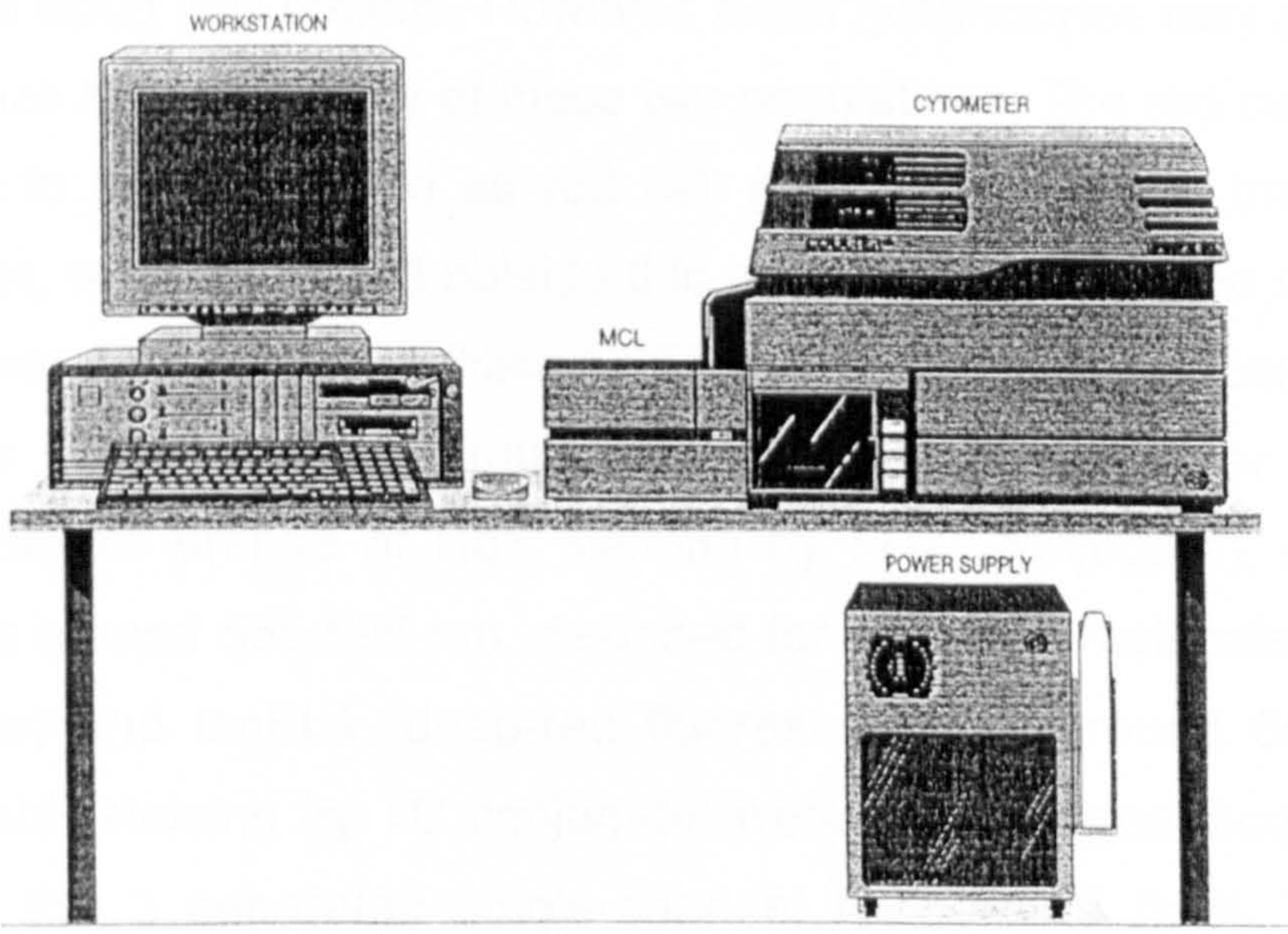


Figure 2.5.1.4 Appearance of Coulter Epics XL-MCL flow cytometer

(From Coulter, Epics XL-MCL, instruction manual). The Coulter Epics XL-MCL has 4 major parts: a work station (a computer controlling all functions, processing and display of results both via the monitor and the print-out from a printer), Cytometer (flow cytometer), Multi-tube carousel loader (MCL) and Power supply (supplying both electric current and positive pressure).

Flow cytometer setting

Gate setting

In the flow cytometer model Coulter Epics XL-MCL (Figure 2.5.1.4) the gate for the red cell area is determined by using forward scatter (FSC) and logarithmic side scatter (logSSC). Firstly, a normal whole blood sample was put through the flow cytometer to get a whole picture of all the different types of blood cell cloud. The red cell cloud was the densest cloud of the picture, since it represents the biggest population in the blood. It could also be recognised as the fourth cloud of both granularity determined by logSS and size determined by FSC levels after granulocyte, monocyte and lymphocyte populations respectively. After the red cell cloud has been recognised, the other clouds were eliminated using the fast set programme of the computer to enhance the signals of both logSS and FSC until only the red cell cloud was present. However, a few lymphocytes may present, due to the similar size and granularity of these two populations. The red cell cloud was then assigned to be recognised as red cell gate. Every cell in this area was analysed further, while every cell outside this area was ignored. The red cell gate was put into histogram number 1 (Hist. 1). Then logarithmic fluorescent channel 1 (logFL1, green fluorescence at around 505-545 nm, designed for either RNA staining by Thiazole orange or HbF staining by FITC conjugate), logFL2 (red fluorescence at around 555-595 nm, designed for adhesion molecule staining by RPE conjugate) and logFL4 (deep-red fluorescence at around 655-695 nm, designed for HbF staining by TC conjugate) were set for signal acquisition and were put into the 3 separated single-channel histograms (Hist. 2, 3 and 4 respectively). Any cell in the red cell gate that emits any of these 3 fluorescent signals, will be recognised and counted as a positive cell in an appropriated histogram according to its fluorescence. After that the 3-pair combinations of double-colour signals (logFL1 vs logFL2, logFL1 vs logFL4 and logFL2 vs logFL4) were put into the 3 separated double-channel histograms (Hist. 5, 6 and 7 respectively). The combination of all 3-colour signals was put into a triple-colour or prism histogram (Hist 8). This histogram showed the cell numbers in all combinations of the 3-colour signals (i.e. ---, +--, --+, +-+, -+-, ++-, -++ and +++ for

logFL1 (green), logFL2 (red) and logFL4 (deep-red) respectively. A high flow rate and auto-stop at 50,000 events (particles or cells) within 360 seconds were used. (Refer to Coulter Epics XL-MCL instruction manual).

Colour compensation

Interfering signals among these 3 colours were eliminated by using the fast colour compensation programme of the computer. Colour compensation can be set by putting one colour staining cells through the machine, which was then scaled up or down the fast compensation until no signal was present outside its channel. For example, with the FITC-staining cells the signals should present only in the green area not in the red or deep-red area adjacent to it. The compensation has to be set like this for all 3 colour used. (Refer to Coulter Epics XL-MCL instruction manual).

Cut-off setting

The base line auto-fluorescence for non-specific binding was set at less than 0.3% (which is greater than mean plus 3 S.D. of negative control population) using samples which were either unstained or stained with irrelevant isotype control antibodies. This setting is recognised as a cut-off (point or line as appropriate) separating positive from negative populations. Any cell producing a fluorescent signal higher than this level will be counted as a positive cell. The cut-off at 0.3% was the best fit among the potential cut-off at 1%, 0.5%, 0.3% and 0.1%, since it showed the best agreement between theoretical and observed values. The various theoretical values of $\%F^{+}$cells were obtained from the known percentages of the mixture of foetal red cells in adult red cells and the various theoretical values of %reticulocytes were obtained from reticulocyte count data from Sysmex SE-9500 blood cell analyser. Unfortunately, the theoretical values of percentages of adhesion molecule expressing cells were unavailable. None-the-less, the machine allowed the use of only one cut-off value that applied to all histograms. However, the cut-off value at 0.3% was the best fit for all histograms. (Refer to Coulter Epics XL-MCL instruction manual).

Getting the results

In conclusion, the flow cytometer settings were used universally for all flow cytometric measurements in this thesis, since it covered all single-, double- and triple-colour channel histograms in one setting. In case of single- or double-colour flow cytometric measurements, only the appropriate histograms were considered to be the actual results. For examples, in the single-colour staining of F^+cells by FITC conjugate only Hist.1 and 2 were the appropriate results and for double-colour staining of reticulocytes by TO vs F^+cells by TC conjugate only Hist. 1, 2, 4 and 6 were the appropriate results. For the triple-colour staining all histograms were the appropriate results. (Refer to Coulter Epics XL-MCL instruction manual) (Figure 2.5.1.5)

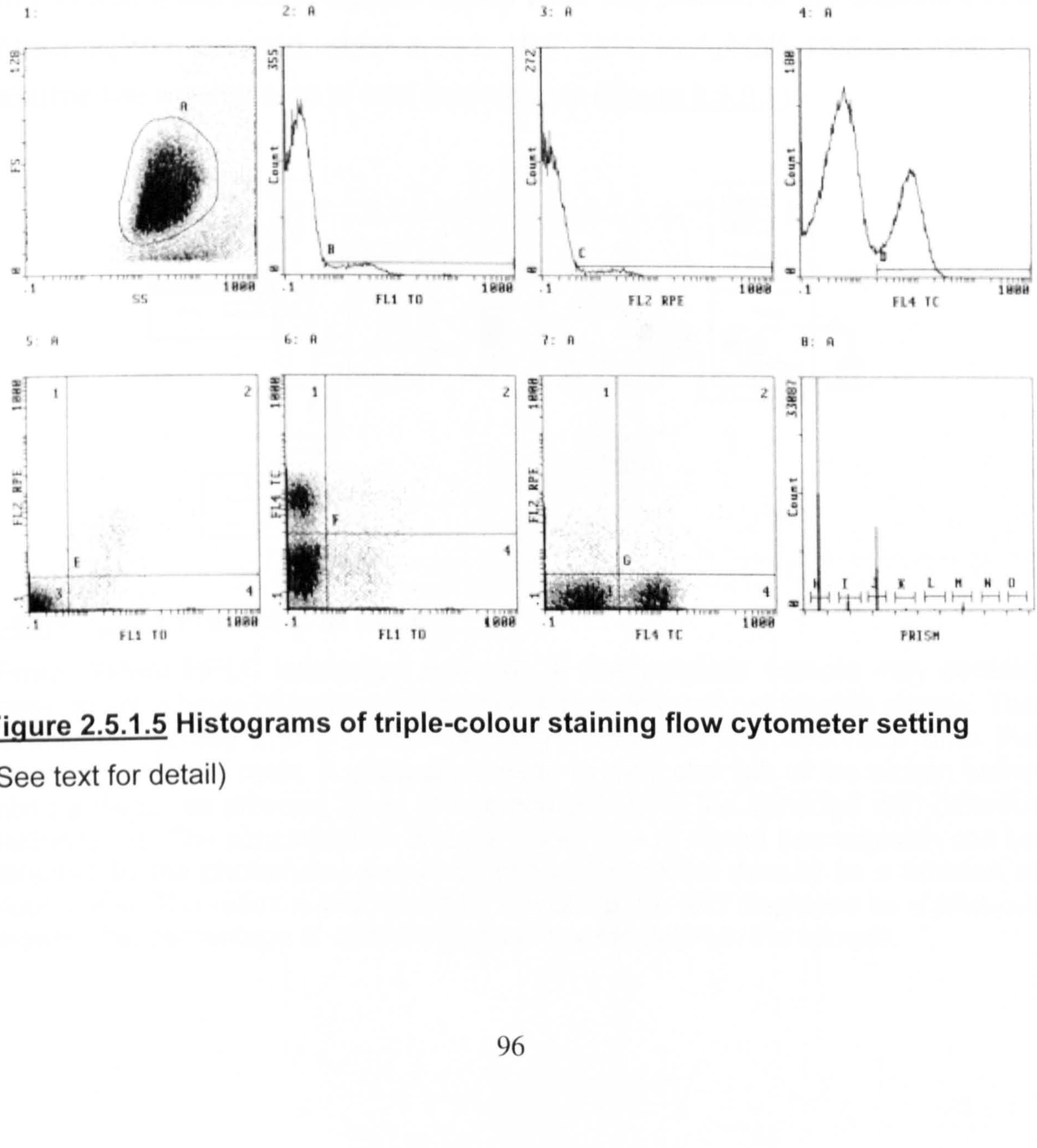


Figure 2.5.1.5 Histograms of triple-colour staining flow cytometer setting

(See text for detail)

2.5.2. High pressure liquid chromatography (HPLC)

Principles of HPLC for quantitative assay of haemoglobin

High Pressure Liquid Chromatography (HPLC; BioRad Variant, USA) was employed for haemoglobin typing and quantitation by using a cation-exchange cartridge on haemolysate injected into the analysis stream according to a previously determined protocol (Tan *et al.*, 1993). In this system, two dual-pistol pumps and a pre-programmed gradient are used to control the elution buffer mixture passing through the analysis cartridge. As the ionic strength of the mixture increases, more strongly retained haemoglobin eventually elutes from the cartridge. Changes in absorbance of the eluate are monitored at 415 nm (with 690 nm correction) and plotted against elution time. The amount of the different types of haemoglobin detected, which include HbF, HbA, HbA_2/HbE, HbS and HbC, is calculated as a percentage of total haemoglobin (Figure 2.5.2.1).

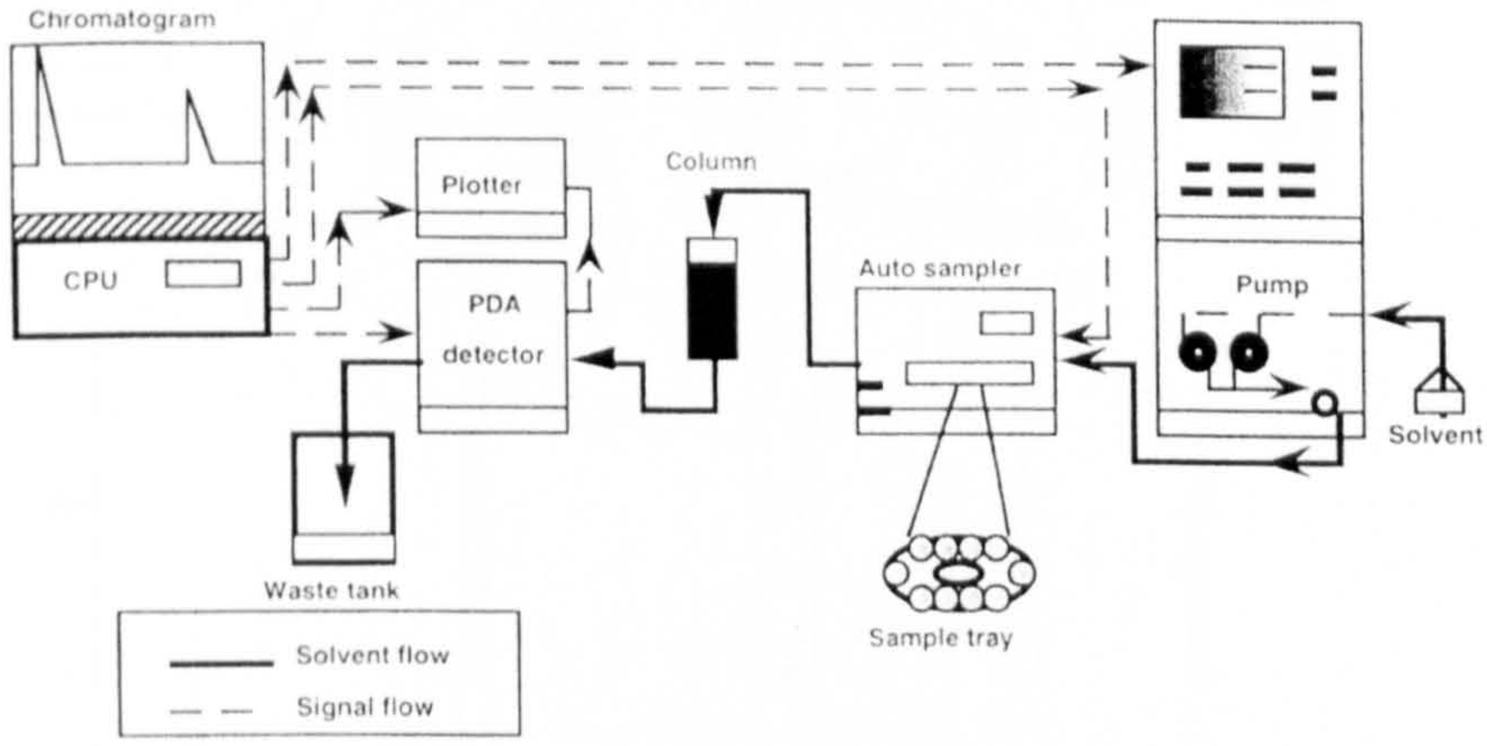


Figure 2.5.2.1 Principles of HPLC

(From Waters HPLC instruction manual). A haemolysate sample may contain many different types of haemoglobin each with a different net electric charge. The sample is injected into a cation-exchange cartridge and absorbed onto the negatively charged resin. A gradual increase in ionic strength of the elution buffer mixture displaces different types of haemoglobin from the cartridge with different elution times. The concentration of a particular type of eluted haemoglobin can be detected by the photometer and displayed as an optical density as a function of elution time. The result is processed by the computer and displayed as a print-out showing the percentage of different types of haemoglobin in the sample.

Foetal haemoglobin assay by HPLC

Five µl of heparinised whole blood was haemolysed with 1 ml haemolysing reagent in 1.5 ml sample vials. The haemolysate was stable for 24 hours at 4°C prior to assay. Two hundred and fifty µl of haemolysate was transferred into a sample vial. The reagents and haemolysate vials were placed into the sample tray in the following order; stat well = haemoglobin primer (a haemolysate standard sample for the beginning of each run), 1st well = deionised water (for blank control), 2nd well = HbA_2/F calibrator (a haemolysate sample of known concentration of HbA2 and HbF for calibrating the machine), 3rd well = normal control (a haemolysate of normal healthy control subject), 4th well = abnormal control (a haemolysate of ß-thalassaemic patient), 5th to nth well = haemolysate samples to be run, n+1th well = normal control, n+2th well = abnormal control and the run commenced. The percentage of HbA, HbA_2/HbE, HbF, HbS and HbC was reported for each sample. (Bio-Rad, Instruction Manual, 1996.) (Figure 2.5.2.2)

ANALYTE ID	%	TIME	AREA
F	4.9	1.16	150456
Unknown 1	0.6	2.20	18699
Ao	1.3	2.36	40489
Unknown 2	0.1	3.82	4155
A2	4.5	3.62	107045
S-WINDOW	89.0	4.46	2712023
	TOTAL AREA		3032787
F	4.9%	A2	4.5%

ANALYTE ID	%	TIME	AREA
F	2.4	1.15	53226
P2	3.8	1.40	93450
P3	3.0	1.81	74331
Ao	39.0	2.46	953162
A2	4.1	3.70	85141
S-WINDOW	48.1	4.52	1176121
	TOTAL AREA		2435431
F	2.4%	A2	4.1%

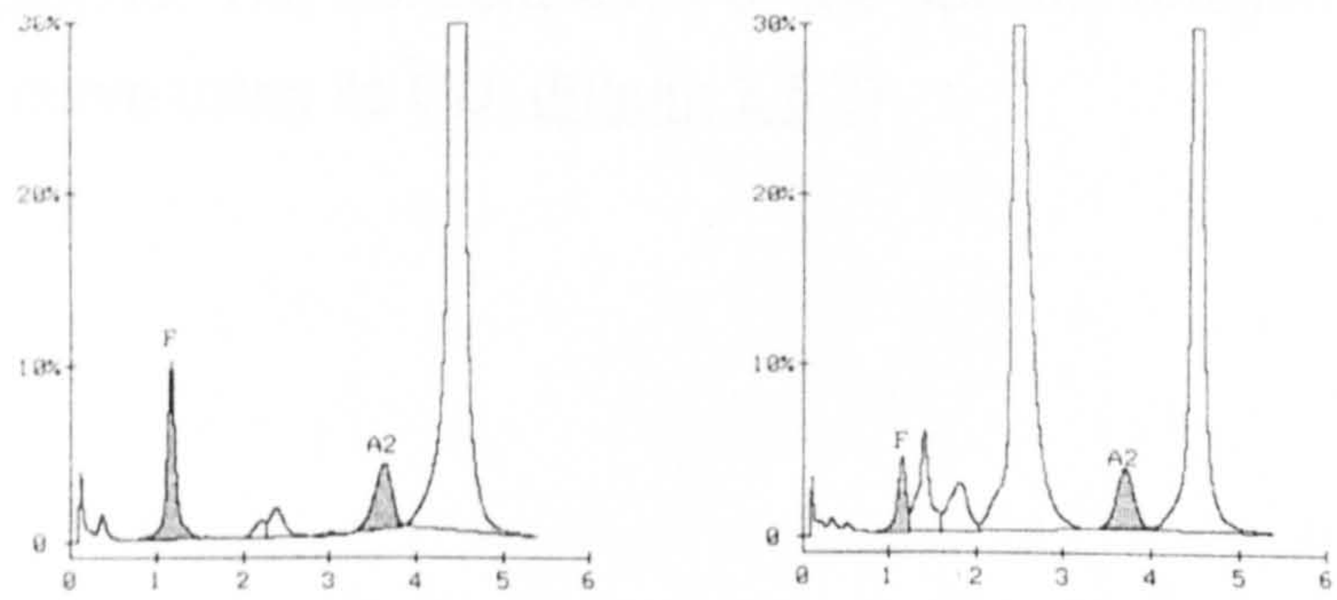


Figure 2.5.2.2 Print-out results from BioRad HPLC

The percentage of different types of haemoglobin in the sample and optical density plotted against elution time are shown. Left is a sickle cell patient (homozygous HbS or SS). Right is sickle cell trait (heterozygous HbS and HbA or AS) or a SS patient with approximately 50% normal HbA from blood transfusion. To differentiate these two conditions, the transfusion history is essential.

2.5.3. Enzyme linked immuno-sorbent assay (ELISA)

Principles of ELISA

Vascular endothelial growth factor (VEGF), the soluble form of endothelial selectin (sE-Selectin), the soluble form of vascular cell adhesion molecule 1 (sVCAM-1), erythropoietin (Epo), ß-thromboglobulin (BTG) and platelet factor 4 (PF4) were all measured using antigen capture ELISA or sandwich ELISA. Inside the wells of a standard 96 well ELISA plastic plate are pre-coated with a specific monoclonal antibody against the antigen of interest. Test samples are added and the relevant antigen in the samples is captured by the antibody on the well. Unbound antigen in the sample is then washed out and the polyclonal antibody against another epitope of the same antigen conjugated to an enzyme is added to bind to the antigen. Unbound antibody is then washed out and a chromogen substrate is added to react with the enzyme on the bound antibody to produce a coloured product. The enzymatic reaction is then stopped at a certain of time. The optical density (OD) is measured by an ELISA reader. A series of different known concentrations of the specific antigen are assayed within the same run as the test samples and their OD are used to create a standard curve, where concentration and OD are plotted. The concentration of the specific antigen in the samples is read from the curve using its OD. (Figure 2.5.3)

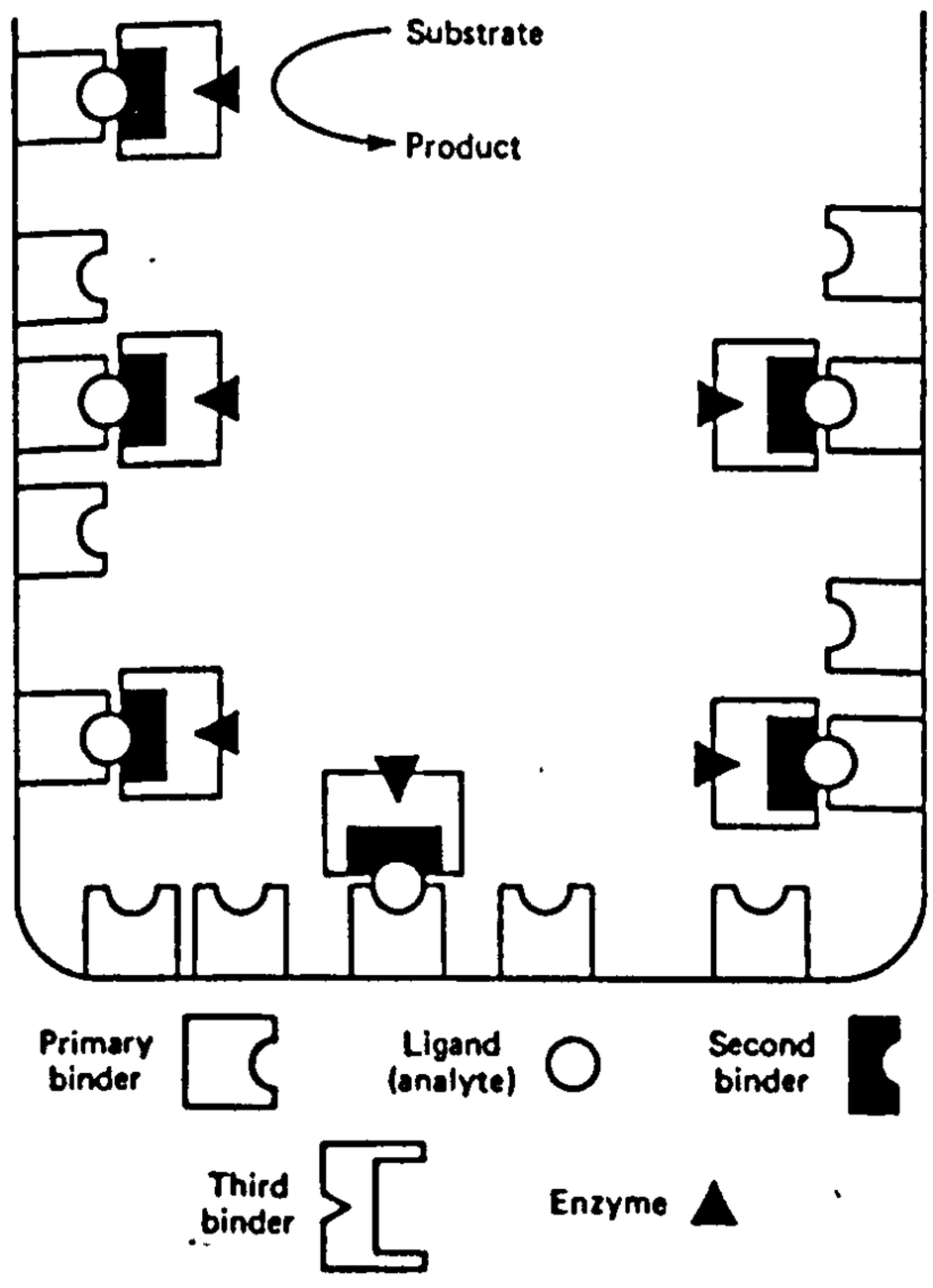


Figure 2.5.3 Principles of sandwich ELISA

(From Stites 1994) See text for detail.

Vascular endothelial growth factor (VEGF) assay

One hundred µl of assay diluent was dispensed into each well, which was pre-coated with monoclonal anti-human VEGF, and 100 µl serum or plasma was added, mixed well and incubated at room temperature (RT) for 2 hours (hr). The wells were washed 3 times with washing buffer and 200 µl polyclonal anti-human VEGF conjugated to horse-radish peroxidase (HRP) was added and incubated for 2 hr at RT. The wells were then washed 3 times, 200 µl tetramethyl benzidine (TMB) substrate solution was added and incubated for 25 minutes (min) at RT in the dark, after that 50 µl stop solution (2N sulphuric acid) was then added. The absorbance was read at 450 nm within 30 min. A series of 4 known concentrations of VEGF were assayed within the same run along with the test samples to create a

standard curve, then the absorbances of the test samples were used for obtaining their VEGF concentrations.

Erythropoietin (Epo) assay

One hundred µl assay diluent was dispensed into each well, which was pre-coated with monoclonal anti-human Epo, and 100 µl serum or plasma was added, mixed well and incubated at RT for 2 hr. The liquid was then carefully aspirated and 200 µl polyclonal anti-human Epo conjugated to HRP was added and incubated for 2 hr at RT. The wells were then washed 4 times with washing buffer and 200 µl TMB substrate was added and incubated for 25 min at RT in the dark, 100 µl stop solution (2N sulphuric acid) was added. The absorbance was read at 450 nm within 30 min. A series of 4 known concentrations of Epo were assayed within the same run along with the test samples to create a standard curve, then the absorbances of the test samples were used to obtain their Epo concentrations.

Soluble endothelial selectin (sE-selectin) assay

Serum or plasma samples were diluted 1:20 with assay diluent. One hundred µl polyclonal anti-human E-Selectin conjugated to HRP was dispensed into each well, which was pre-coated with monoclonal anti-human E-Selectin, and 100 µl diluted serum or plasma was added, mixed well and incubated at RT for 1.5 hr. The wells were then washed 6 times with washing buffer and 100 µl TMB substrate was added and incubated for 30 min at RT in the dark, 100 µl stop solution (2N sulphuric acid) was added. The absorbance was read at 450 nm within 30 min. A series of 4 known concentrations of sE-Selectin were assayed within the same run along with the test samples to create a standard curve, then the absorbances of the test samples were used to obtain their sE-Selectin concentrations.

Soluble vascular adhesion molecule 1 (sVCAM-1) assay

Serum or plasma samples were diluted 1:50 with assay diluent. One hundred µl polyclonal anti-human VCAM-1 conjugated to HRP was dispensed into

each well, which was pre-coated with monoclonal anti-human VCAM-1, and 100 µl diluted serum or plasma samples was added, mixed well and incubated at RT for 1.5 hr. The wells were then washed 6 times with washing buffer and 100 µl TMB substrate was added and incubated for 20 min at RT in the dark, 100 µl stop solution (2N sulphuric acid) was added. The absorbance was read at 450 nm within 30 min. A series of 4 known concentrations of sVCAM-1 were assayed within the same run along with the test samples to create a standard curve, then the absorbances of the test samples were used to obtain their sVCAM-1 concentrations.

ß-Thromboglobulin (BTG) assay

Serum or plasma samples were diluted 1:10 with assay diluent. Two hundred µl diluted serum or plasma was dispensed into each well, which was pre-coated with monoclonal anti-human BTG, and incubated at RT for 1 hr. The wells were then washed 5 times with washing buffer and 200 µl polyclonal anti-human BTG conjugated to HRP was added and incubated for 1 hr at RT. The wells were then washed 5 times and 200 µl orthophenylene diamine dihydro chloride (OPD) substrate was added and incubated for exactly 3 min at RT in the dark, 100 µl stop solution (1M hydrochloric acid) was added. The absorbance was read at 490 nm within 30 min. A series of 4 known concentrations of BTG were assayed within the same run along with the test samples to create a standard curve, then the absorbances of the test samples were used to obtain their BTG concentrations.

Platelet factor 4 (PF4) assay

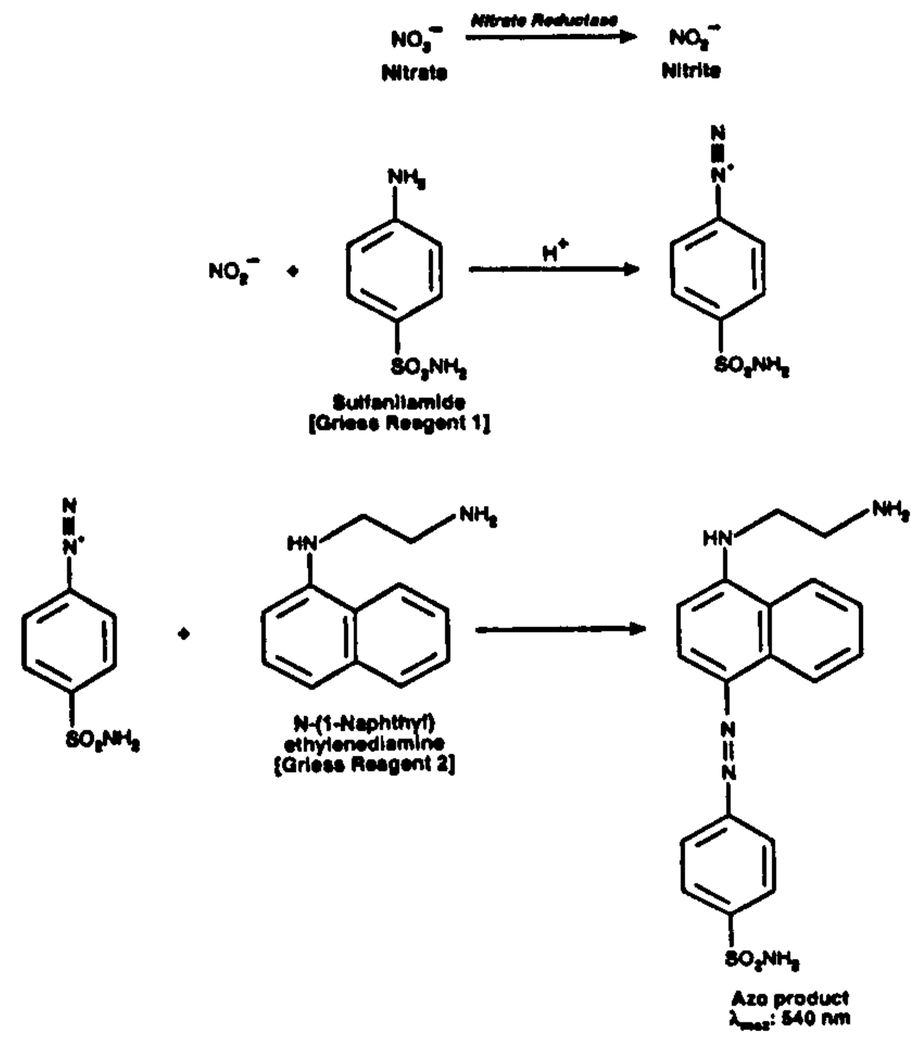
Serum or plasma samples were diluted 1:5 with assay diluent. Two hundred µl diluted serum or plasma was dispensed into each well, which was pre-coated with monoclonal anti-human PF4, and incubated at RT for 1 hr. The wells were then washed 5 times with washing buffer and 200 µl polyclonal anti-human PF4 conjugated to HRP was added and incubated for 1 hr at RT. The wells were then washed 5 times and 200 µl OPD substrate was added and incubated for exactly 3 min at RT in the dark, 100 µl stop solution (1M hydrochloric acid) was then added.

The absorbance was read at 492 nm within 30 min. A series of 4 known concentrations of PF4 were assayed within the same run along with the test samples to create a standard curve, then the absorbances of the test samples were used to obtain their PF4 concentrations.

2.5.4. The Griess Reaction

Principles of the Griess Reaction

Nitric oxide metabolites (NOx) can be measured colorimetrically using the Griess reaction (Nims *et al.*,1995). Serum and plasma samples must be filtered through an ultra-filter with MW cut-off of 30 kDa to avoid interference from coloured proteins, for example bilirubin and haemoglobin, since there is no washing step. Nitrates in the sample are converted to nitrites by nitrate reductase and its co-factors. The nitrite is coupled to Griess reagent 1 (sulphanilamide) and Griess reagent 2 (N-(1-Naphthyl) ethylenediamine) to form a deep purple azo-product. The optical intensity (OD) of the final product is directly correlated with the concentration of total nitric oxide metabolites (NOx or nitrates plus nitrites). Serial dilution of known nitrite concentration solutions and their OD are used to create a standard curve. The concentration of NOx in the samples is obtained from the curve using their OD. (Figure 2.5.4)



Chemistry of the Griess Reagents

Figure 2.5.4 Principles of the Griess Reaction

(From Cayman Chemical, NO assay instruction manual) See text for detail.

Nitric oxide metabolites (NOx) assay

Serum or plasma samples were diluted 1:4 with assay diluent, then they were ultra-centrifuged, passing through an ultra-filter with a 30 kDa molecular weight cut-off (Whatman Inc., USA.) to eliminate interference from high molecular weight protein. Eighty µl of filtrated sample was then filled into the well of a 96 well plastic plate. Ten µl each of enzyme co-factor and nitrate reductase were added and incubated at RT for 3 hr. Griess Reagent R1 and Griess Reagent R2 (50 µl each) were added and the colour was allowed to develop for 10 min. The absorbance was read at 540 nm within 30 min and the concentration of NOx in each sample was obtained from standard curve, which was created by assaying a series of 7 known concentrations of NOx within the same run along with the samples.

2.5.5. Platelet activation experiment

Platelet rich plasma from citrate blood samples form 6 normal control subjects were divided into 2 aliquots of 0.5 ml each. One aliquot was activated with 100 µl thrombin activating protein (TRAP at a final concentration of 80 µM) and 100 µl PBS was added into another aliquot and then incubated at RT for 30 min. These two aliquots were centrifuged at 1500g for 10 min. The platelet poor plasma supernate with platelet activation (Platelet+) and without platelet activation (Platelet-) were collected and stored in –70°C for assaying of VEGF, NOx, sE-Selectin, sVCAM-1, BTG and PF4.

2.5.6. Reticulocyte culture

Twenty five µl EDTA fresh whole blood from 8 sickle cell patients was cultured in 25 cm^2 flasks with 25 ml of RPMI-1640 culture medium in 5% CO_2 at 37°C for 15 days. Assays for AM expression on reticulocytes and red cells were carried out on cultured red cells on days 0 (within the first hour), 1, 2, 3, 4, 5, 10 and 15 of culture respectively.

2.5.7. Calculation of enrichment ratio (ER) and adhesion molecule depletion ratio (AMDR)

The enrichment ratio (ER) and adhesion molecule depletion ratio (AMDR) are defined as the ratio of the proportion of a specific population within total mature red cells to the proportion of the same population within total reticulocytes. In order to examine the effect of AM expression and HbF containing on red cell survival; ER and AMDR have been compared among the different groups of sickle cell patients and normal control subjects.

F^+cell enrichment ratio estimation

The ratio of $\%F^+$mature red cells to $\%F^+$reticulocytes has been previously described as an enrichment ratio (Dover, 1978a). The formula for ER is shown below.

$$ER = \frac{\text{Number of } F^{+}\text{mature red cells / Total number of mature red cells}}{\text{Number of F+reticulocytes / Total number of reticulocytes}}$$

Adhesion molecule depletion ratio estimation

The ratios of $\%AM^{+}$mature red cells to $\%AM^{+}$reticulocytes were calculated and called the adhesion molecule depletion ratio (AMDR). The formula for AMDR is shown below:

$$AMDR = \frac{\text{Number of } AM^{+}\text{mature red cells / Total number of mature red cells}}{\text{Number of } AM^{+}\text{reticulocytes / Total number of reticulocytes}}$$

Three-circle-overlapping diagram for triple-colour staining results

In order to calculate percentages of many different sub-populations and their ER or AMDR more easily, the numbers of each red cell sub-population from triple-colour staining flow cytometry was put into the relevant area of three-circle-overlapping diagram (Figure 2.5.7).

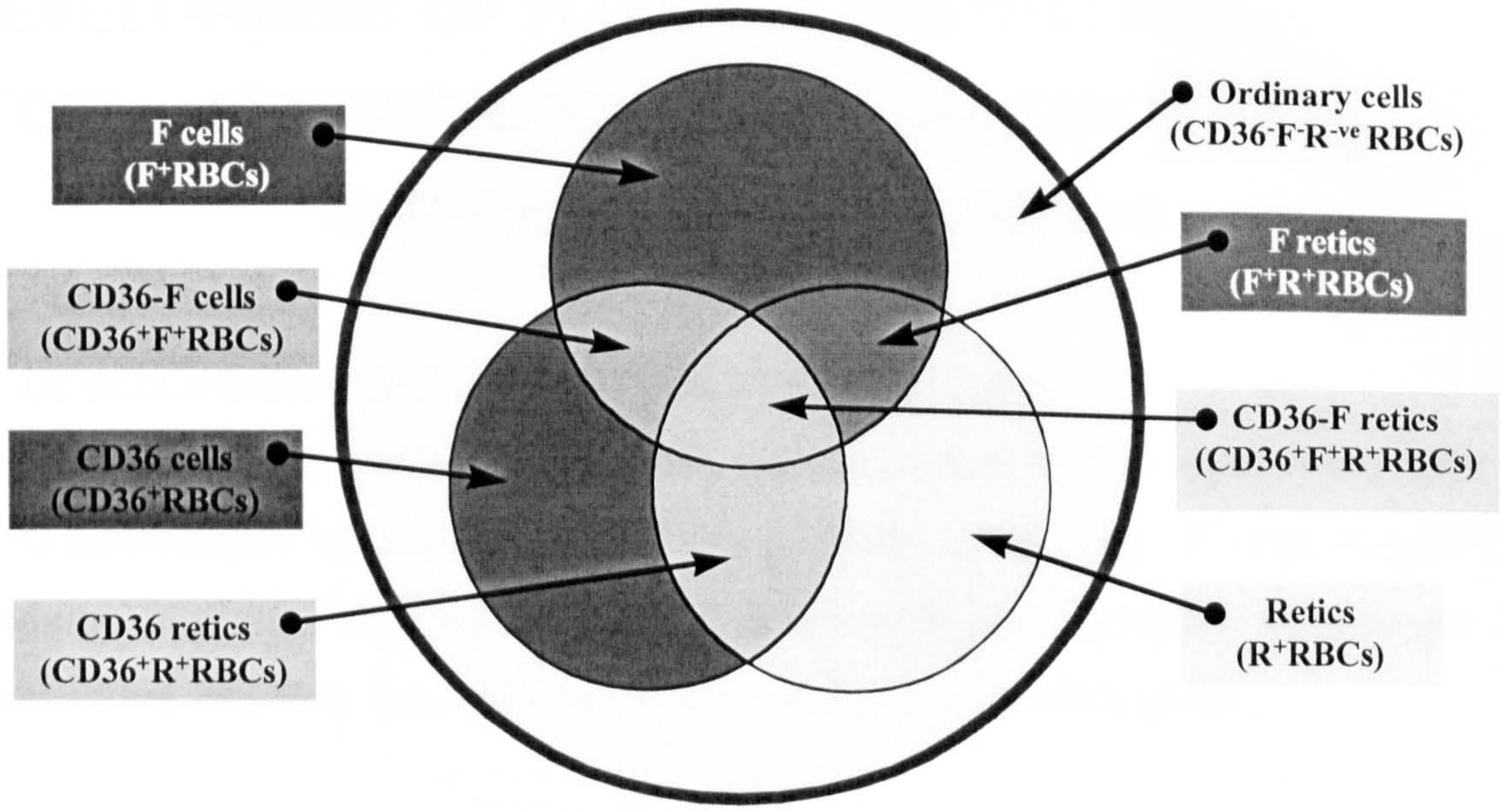


Figure 2.5.7 Three-circle-overlapping diagram for triple-colour staining

Each circle represents the positive population of each colour: green fluorescence for RNA or reticulocytes (R^+), red fluorescence for adhesion molecule expressing cells (AM^+) and deep-red fluorescence for HbF containing cells or F^+cells (F^+). Each overlapping area represents each double-colour positive population between the adjacent fluorescence. The middle area represents the triple-colour positive population.

2.6. STATISTICS AND CALCULATION

All statistics are shown as the mean ± standard error of the mean (SEM) unless stated otherwise. The means of two groups were compared using a student's unpaired t-test, and the means for pre- and post-treatment were compared using student's paired t-test. The correlation coefficient (r) between two parameters was calculated to estimate the degree of correlation. All significant levels are shown using 95% confidence intervals or p value less than 0.05.

CHAPTER 3

DEVELOPMENT OF FLOW CYTOMETRIC METHODS FOR F^+CELL, F^+RETICULOCYTE AND ADHESION MOLECULE EXPRESSING CELL ASSAYS

3.1. DEVELOPMENT OF FLOW CYTOMETRIC METHODS

In this chapter, the development and validation of flow cytometric methods for determination of the proportion (in percent) of F^+cells, F^+reticulocytes and adhesion molecule expressing cells is described. The possible advantages and applications of using flow cytometry for these purposes are given.

3.1.1. Methods for HbF and F^+cell assays

HbF assay

Foetal haemoglobin (HbF) can be measured by a number of different methods. Some methods are based on acid or alkali resistance of HbF compared to HbA and HbA_2. In the alkali denaturation test (Betke et al. 1959), HbF is determined by using an alkali solution to precipitate other Hbs out of the haemolysate. The rest is HbF, which resists alkali denaturation. HbF is then measured by spectrophotometric method (at 540 nm) and compared to the total Hb in original haemolysate. This method has generally good reproducibility within the same laboratory but in different laboratories, it is highly variable. Therefore each laboratory should set their normal range with a sample size over 50. However, an upper limit for normal values of either 1% or 2% is acceptable (Chanarin, 1989). No single method is suitable for measuring HbF levels across the whole range of 0-100% but the alkali denaturation test is the most widely used because of its simplicity. However, its tendency to overestimate at the very low levels and underestimate at the high levels has to be taken into account. Values for %HbF within the range of 5–50% are more reliable by this method. Electrophoresis (Steiman *et al.*, 1985) and isoelectric focusing (Leary *et al.*,

1983) can be employed to determine HbF based on differences in net electric charge on the different types of Hb (Manca *et al.*, 1986). Since HbF has a greater net positive charge than HbA, it moves a longer distance than HbA and HbA_2 in electrophoresis with citrate agar at pH 6.0. However, electrophoresis is normally only a qualitative assay for a haemoglobinopathy primary diagnosis and is not suitable for quantitative work. Immunodiffusion (Ouchterlony) using anti-HbF antibody is simple, sensitive but time consuming. This method is reliable for the quantitation of HbF at very low levels (under 2%) (Marti *et al.*, 1983). Column chromatography is reliable at higher HbF levels (over 40%), but the technique is cumbersome and time-consuming (Rajantie *et al.*, 1992). Radioimmuno assay is a sensitive method, which can detect very low levels (in picograms) of HbF, but needs expensive equipment (James *et al.*, 1980). High performance liquid chromatography (HPLC) is convenient, sensitive and reproducible, but also needs expensive equipment (Huisman *et al.*, 1981; Tan *et al.*, 1993). The lowest detection limit (sensitivity) for %HbF of Bio-Rad HPLC is ~1% (Waters, et al. 1996).

All of these methods can give an assay of total foetal haemoglobin (HbF) but give no indication of the distribution of HbF within the cells. This information is important in the interpretation of the relevance of HbF levels. For example; if 20% of cells have 100% HbF in sickle cell patients, the impact on the disease may be quite different from 100% of cells having 20% HbF. Therefore knowledge of the distribution of HbF is potentially valuable. Determination of F^+cells in the circulation is also used as a way of measuring foeto-maternal haemorrhage in the management of haemolytic disease of the new-born (HDN).

F^+cell assay

F^+cell assay is in common use in clinical practice because it is important to quantitate the haemorrhage of foetal cells (measured as F^+cells) into maternal circulation in detecting the risk of HDN. The method traditionally used for this purpose is the Kleihauer test (Kleihauer, 1957). In this test, HbF resists acid-elution and remains in the cells, while the other types of Hb are eluted out leaving

cells of a ghost appearance after staining, in contrast to the dark positive appearance of F^+cells. The limitations of the Kleihauer test include not sensitive, subjective and time consuming. Using anti-HbF antibody conjugated fluorescent dye or immunofluorescent staining (Osterhout *et al.*, 1996) on a fixed blood smear is more sensitive, but is still dependent on technical skills. These two methods require manual evaluation on fixed blood smears. The later method is easier, more rapid but still uses a blood smear, which is subjective depending on the morphological skill of the technician. These methods give information on about 1-7% of positive cells among a large number of negative cells. So a large number of total cells need to be counted manually to achieve statistical power, and this is time-consuming (Wood *et al.*, 1975). Normally the total count of these methods is 1,000 or 5 high power fields (x400), which have approximately 200 cells per field in a well spread and non-overlapping area of the blood smear. The sensitivity depends on the technical skill of the observer. Some observers recognise F^+cells better than others and furthermore, when F^+cells are scarce, a larger number of cells need to be analysed.

Another way of measuring HbF containing red cells (F^+cells) is by flow cytometry. Previously described flow cytometric methods have required stringent conditions of cold permeabilisation with acetone at -15 to -20 °C, which are not easy to handle and it takes time to prepare the samples (Thorpe *et al.*, 1994). These methods are therefore not easy to perform. However, flow cytometry examines up to 50,000 cells within a few minutes and does not require much technical skill. This advantage makes it one of the most powerful analytical tools in biological field.

Relationship of % HbF to % F^+cells

The proportion of F^+cells in normal adults is approximately 1-5% by the Kleihauer test (McKenzie, 1996). When the proportion is less than 1%, there is an intrinsic error therefore a larger number of cells needs to be counted to overcome the error (Dacie & Lewis, 1970). Immunofluorescent staining gives a value of 0.2-7% which is comparable to flow cytometry (Wood *et al.*, 1975). The latter two

techniques use the same principle but differ in the detection. However, flow cytometry is much more reliable than microscopic immunofluorescence, because more cells are analysed.

It has been estimated that the quantity of HbF per F^+cell is approximately 5 pg/cell (Wood *et al.*, 1975). This value has been calculated from %F^+cells obtained from the acid elution test, %HbF obtained from the alkali denaturation test and MCH obtained from full blood count (FBC) data. The formula is (HbF/F^+cells = MCH x %HbF / %F^+cells) (Maier-Redelsperger *et al.*, 1994). Microscopic-single-cell-radial immunodiffusion also showed similar levels of HbF/F^+cells at 4-8 pg/cell (Boyer *et al.*, 1975), which was a comparable value to that obtained using the calculation method. The %F^+cells correlates well with %HbF in samples containing up to 5% HbF (Dacie & Lewis, 1991). This correlation suggests that in adult populations, the mean levels of HbF per F^+cell remain almost constant. However, the amount of HbF per F^+cell required for a cell to be recognised as a F^+cell may vary with different techniques. Levels of HbF at 1pg/cell or approximately 3.5% of MCH are possibly high enough for sensitive methods such as immunofluorescence and flow cytometry. However, the acid elution test may need more HbF per cell than that to be recognised as a F^+cell (Dacie & Lewis, 1991). Therefore, the %HbF and %F^+cells are not the same parameters but their relationship is very close. The different levels of %HbF and %F^+cells might influence the clinical outcomes in sickle cell disorders to different degrees.

HbF and F^+cell distribution

Normal adult blood contains 3 types of haemoglobin (96-98% HbA, 1.5-3.2% HbA_2 and 0.5-2.0% HbF). Adult haemoglobin (HbA) appears in normal foetuses at about 5-10% from 6-12 weeks of gestation onward and increases sharply just before birth. The β-globin gene is expressed at low levels in early foetal life, but the main switch to adult haemoglobin occurs 3-6 months after birth, when synthesis of the γ-globin chain is largely replaced by the β-globin chain. Methylation of the gene is thought to be a possible regulation of the

expression (expressed genes are usually hypomethylated and *vice versa*) (Hoffbrand, 1993).

In normal adults, 0.5-2.0% of haemoglobin is HbF (McKenzie, et al. 1996) and this is distributed in only 1-7% of red cells, termed F^+cells (Wood *et al.*, 1975; Rochette *et al.*, 1994; Thorpe *et al.*, 1994). In normal subjects, each F^+cell contains about 20% HbF (or 4-7 pg of HbF per 30 pg of MCH) together with another 80% of HbA. However, in sickle cell patients, the %HbF may be raised to 4-15% and this is distributed within approximately 4-40% F^+cells (Dover *et al.*, 1978b).

The proportion of F^+cells is even higher in sickle cell patients undergoing hydroxyurea (HU) treatment. Up to 10-30% HbF (Saleh *et al.*, 1997) distributed within approximately 35-75% F^+cells (Bridges *et al.*, 1996; Charache *et al.*, 1992). In hereditary persistent of foetal haemoglobin (homozygous HPFH) only HbF is synthesised in the absence of HbA and HbA_2. In other pathological states such as thalassaemia, %HbF and %F^+cells are dependent on phenotype and genotype (Giampaolo *et al.*, 1984; Marcus *et al.*, 1997; Marinucci *et al.*, 1981; Rees *et al.*, 1999). The %F^+cells has also been a valuable parameter for monitoring sickle cell patients undergoing treatment with a HbF inducing agent such as hydroxyurea (Steinberg, 1997; Steinberg *et al.*, 1997).

3.1.2. Flow cytometric assay of %F^+reticulocytes

Reticulocyte count

In order to develop a flow cytometric method for F^+reticulocytes, it is first necessary to have a simple and reliable flow cytometric method for reticulocyte count alone. Reticulocyte count is essential for monitoring the erythropoietic activity of the bone marrow, especially during therapy and therefore it is a routine test in haematology laboratories. Supravital staining with new-methylene blue is traditionally used in routine manual reticulocyte counts. However, staining of RNA inside reticulocytes in suspension using fluorescent dyes such as Thiazole orange (Chin-Yee *et al.*, 1991), Acridine orange (Seligman *et al.*, 1983), Pyronin Y

(Davis & Bigelow, 1990), Thioflavin (Sage *et al.*, 1983) or Auramine O (Davis & Bigelow, 1990; Tichelli *et al.*, 1990) with flow cytometric analysis is more sensitive, reproducible, objective and is now available on automated blood cell analysers. It was important to choose a method of reticulocyte staining which was applicable for double- and triple-colour staining. Thiazole orange (TO) was chosen because it has wavelengths of excitation at 509 and of emission at 533 nm. These wavelengths are available on our flow cytometer (Coulter Epics XL-MCL, see Table 3.1.2). In addition, the TO emission wavelength does not overlap significantly with rhodamine phycoerythrin (RPE, 576 nm) or Tri-color (TC, 670 nm) which were considered as potential candidates for adhesion molecule and HbF staining respectively in the triple-colour staining.

F^+reticulocyte assay

When the response to hydroxyurea is being monitored, an early response can be identified by examining the presence of young cells containing HbF rather than all F^+cells in the total population (Maier-Redelsperger *et al.*, 1998). A method for measuring reticulocytes containing HbF (F^+reticulocytes) was originally developed by Dover (Dover *et al.*, 1978a) using microscopic-single-cell-radial immunodiffusion. However, this method is time consuming, cumbersome, and requires a specialised and experienced technician.

In principle flow cytometry can be employed to measure HbF and RNA content on the same cells, using double-colour staining. Reticulocytes and F^+cells can be assayed by flow cytometry separately as described above. In order to perform a double-colour staining, the fluorescent emissions used for reticulocyte and F^+cell assay should not significantly overlap. The available fluorochromes for RNA (reticulocytes) and monoclonal antibody against human HbF are shown in Table 3.1.2.

The combination of TO for RNA staining and anti-HbF-TC conjugate for HbF staining were selected for the development of double-colour staining flow cytometry for F^+reticulocyte assay. The original method was developed by Dr Bruch H. Davis (personal communication) but was not reported in the literature.

The method used in this thesis was slightly modified and necessary to be evaluated. The method gives quantitative information of F^+reticulocytes (F^+R^+), F^- reticulocytes (F^-R^+), F^+mature red cells (F^+R^-) and F^-mature red cells (F^-R^-). The absolute numbers of these populations can also be estimated using red blood cell count data and the information above.

Table 3.1.2 Availability of fluorochromes for RNA, anti-AM and anti-HbF conjugates

(From Bauer, et al. 1993 and instruction manual of Coulter Epics XL-MCL)

Fluorochrome	Excite* (nm)	Emit (nm)	Colour	Detector (on Epics XL)
RNA Dye				
Thiazole orange	509	533	Green	FL1
Acridine orange	492	550	Orange	None
Pyronin Y	515	580	Orange	FL2
Thioflavin	422	487	Green	None
Auramine O	435	550	Orange	None
Ethidium Bromide	514	602	Red	None
Propidium Iodide	535	615	Red	FL3
Anti-HbF and anti-AM conjugates				
FITC	495	520	Green	FL1
RPE	495 & 564	576	Orange	FL2
TC	495	670	Deep Red	FL4

*Coulter Epics XL-MCL has only one available excitation wavelength at 488 nm (range 458-518 nm).

3.1.3. Adhesion molecule expressing cell assay by flow cytometry

Some adhesion molecules (AMs) such as CD36, CD41 and CD49d are expressed on sickle red cells and are involved in the erythrocyte-endothelium interaction. Recent work has demonstrated the importance of this expression in sickle cell disorders (Moore *et al.*, 1996), because red cells expressing such

molecules are likely to be preferentially adherent to the endothelium. This in turn may contribute to slowing of blood flow in the microvasculature and may initiate vaso-occlusion in sickle cell disease.

The relationship between erythrocyte adhesion molecule expression and the expression of HbF in erythrocytes has not been investigated previously and is potentially important for two reasons. Firstly, it may provide information about the nature of F^+cells in normal individuals. In particular, whether F^+cells express increased, decreased or normal levels of AMs and whether such cells survive normally. Secondly, in sickle cell disorders, whether co-expression of HbF and AMs accounts for the survival advantage of F^+cells compared to F^-cells.

Therefore, flow cytometric methods for measurements of AM expressing cells (single-colour staining), AM expressing F^+cells and AM expressing reticulocytes (double-colour staining) and AM expressing F^+reticulocytes (triple-colour staining) were developed and employed in this thesis to addressed those questions above.

3.2. DEVELOPMENT AND VALIDATION OF SINGLE-COLOUR STAINING

3.2.1. Background

Initial experiments were undertaken to evaluate F^+cell assay by flow cytometry, using a previous described method (Davis *et al.*, 1998). Subsequent experiments were undertaken to improve the fixation/permeabilisation and staining procedures and to reduce the time taken for sample preparation. Prior to the work in this thesis, assay of F^+cells in thalassaemia and sickle disorders had not been undertaken using the assay described below. Calibration and gating of the flow cytometer has been described in chapter 2.

3.2.2. F^+cell assay using the original procedure

In order to adjust the number of red cells in the assay procedure, a full blood count (FBC) was obtained on every blood sample using the Sysmex SE-9500. Approximately 2.5 x 10^7 red cells (the actual sample volume was estimated

from red blood cell count data) were fixed with 1 ml ice-cold 0.05% glutaraldehyde in phosphate buffered saline pH 7.4, vortexed for 15 seconds (sec.), incubated at room temperature (RT) for 10 minutes (min) and then washed twice with PBS. The red cells were then permeabilised by vortexing for 15 sec. with 0.5 ml ice-cold 0.1% Triton X-100 in 0.1% bovine serum albumin in PBS (BSA-PBS) and incubated at RT for 5 min, then washed once with 0.1%BSA-PBS, and re-suspended in 0.5 ml 0.1%BSA-PBS. Ten μl of red cell suspension was then mixed with 20 μl of 1 in 10 diluted mouse monoclonal antibody (IgG1) against human HbF conjugated to fluorescein isothiocyanate (MoAb-HbF-FITC, green fluorescence) and 70 μl 0.1%BSA-PBS, incubated in the dark at RT for 15 min, then washed twice with 0.1%BSA-PBS and finally fixed with 0.5 ml 1% formaldehyde in 0.1%BSA-PBS. The sample was then analysed by flow cytometry or stored at 4°C in the dark for up to 1 week before analysis. An IgG1 conjugated to FITC (isotype control) was used instead of MoAb-HbF-FITC as a negative control for non-specific binding. Using TC instead of FITC conjugate gave similar results (Figure 3.2.2). Gate setting of flow cytometer is described in Chapter 2.

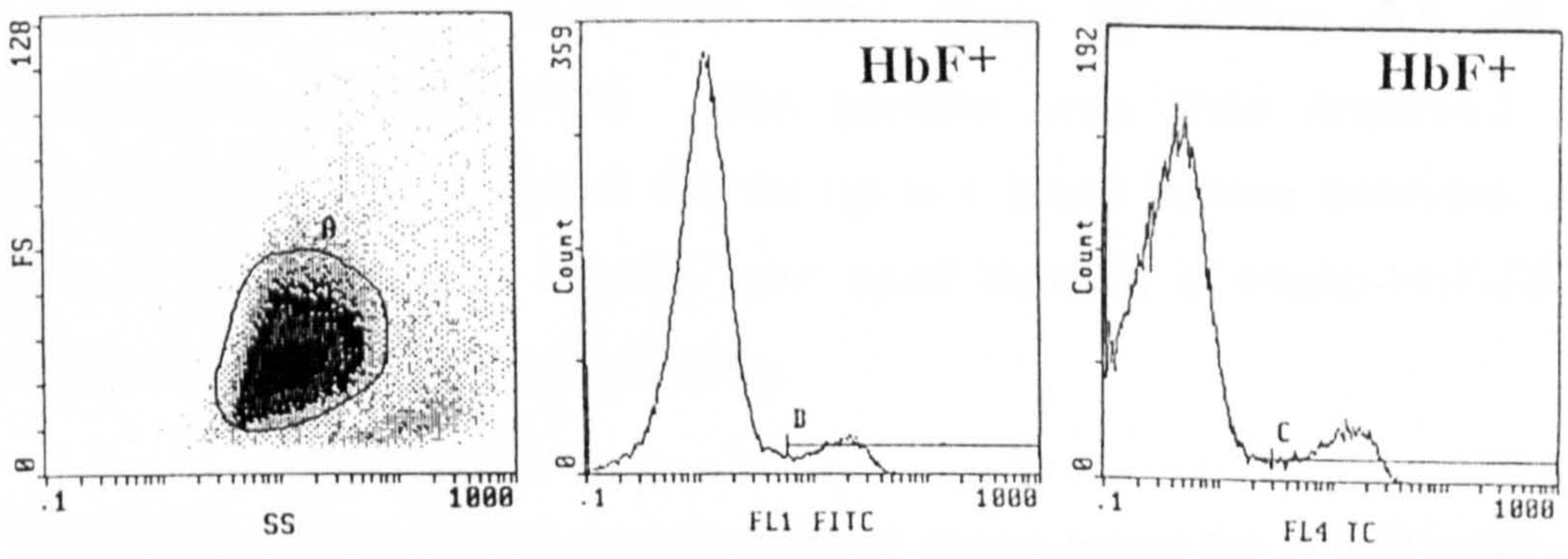


Figure 3.2.2 Flow cytometric histograms of F^+cell assay

The left histogram shows red cell population, which is selected for analysis. The middle histogram shows fluorescent signal from FITC (green). The right histogram shows fluorescent signal from TC (deep-red)

3.2.3. F^+cell assay using simplified procedure

The original procedure for the F^+cell assay has many washing or centrifugation steps and the sample volume to be used in the assay has to be calculated from red cell count data. In order to save time and make the procedure more convenient, the procedure was simplified. Firstly, the samples were adjusted to an approximately 45-50% haematocrit (Hct) by spinning the sample down for 10 minutes at 250 g at 4°C, removing the plasma, then diluting the packed red cells with an equal volume of Alsever's solution : PBS (1:1) mixture. Using this technique the sample volume is constant and no calculation is required. A second advantage is that the washing steps were unnecessary.

Twenty µl of adjusted blood sample as above was fixed with 1 ml of ice-cold 0.05% glutaraldehyde in PBS, vortexed for 15 sec and incubated at RT for 10 min. One hundred µl of the mixture was then vortexed for 15 seconds with 400 µl of ice-cold 0.1% Triton X-100 in 0.1%BSA-PBS and incubated at RT for 5 minutes for permeabilisation. Ten µl of this mixture (final dilution is approximately 1 in 255) was then vortexed with 20 µl of a 1 in 10 dilution of MoAb-HbF-FITC. Then 70 µl of 0.1%BSA-PBS was added and the mixture was incubated in the dark at RT for 15 min. The sample was then fixed again by adding 0.5 ml of 1% formaldehyde in 0.1%BSA-PBS. The sample was then analysed by flow cytometry, but could be stored at 4°C for up to 1 week before analysis. An IgG1 conjugated to FITC (isotype control) was used instead of MoAb-HbF-FITC as a negative control for non-specific binding.

3.2.4. Comparison of original and simplified procedures for F^+cell assay

Study design

Four blood samples containing various known % of F^+cells (5, 25, 50, and 100% respectively) were prepared by mixing cord-blood-red cells with adult-blood-red cells and assayed for %F^+cells using both original and simplified procedures (section 3.2.2 and 3.2.3 respectively). Twelve blood samples from 8 sickle cell and 4 thalassaemic patients were assayed in parallel between

original and simplified procedures. Samples from 12 patients were analysed to compare the results obtained using the original and simplified procedures.

Results

Using the original procedure on the mixtures containing known %F^+cells of 5, 25, 50 and 100% respectively, the results were 5.8, 23.2, 52.5 and 99.3% respectively. Using the simplified procedure on the same samples, the results were 5.7, 21.8, 52.5 and 98.5% respectively. The positive cut-off was set at 0.3% in both procedures. A clear distinction between F^+cells and F^-cells are shown in Figure 3.2.4.1.

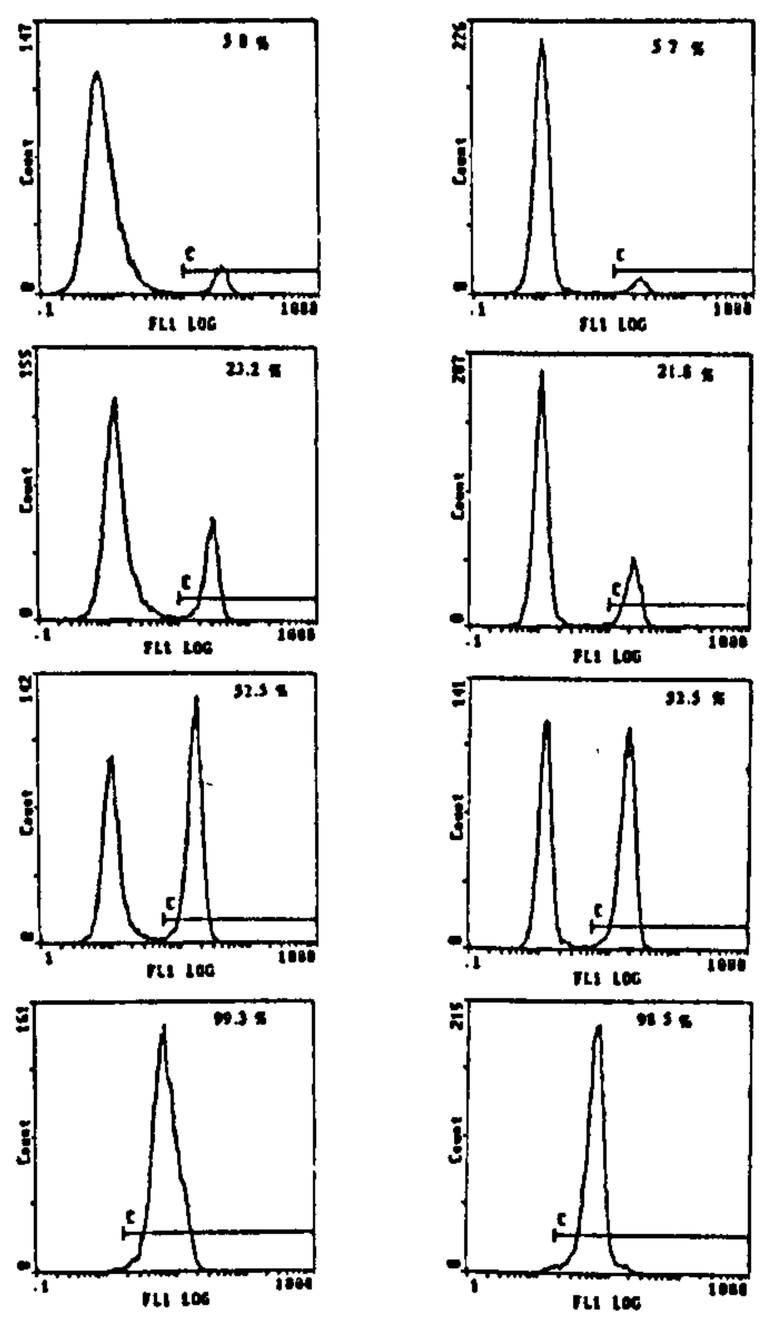


Figure 3.2.4.1 Comparison histograms of original and simplified procedures

Four mixtures of cord blood and adult red cells containing 5, 25, 50 and 100% cord blood red cells respectively, were assayed for %F^+cells in parallel using both the original (left) and simplified (right) procedures. Using the cut-off setting at 0.3% above the negative control population, the difference between positive and negative population was clear in both procedures. There was no difference between these two set of results.

When samples from 12 patients were analysed to compare the results obtained using original and simplified procedures, the mean %F^+cells were 23.92±2.28% and 23.73±6.19% respectively without any significant difference. These results are shown in individual patients in Table 3.2.4.

Table 3.2.4 Comparison of F^+cell results of original and simplified procedures

Sample Number	Diagnosis	Original Procedure	Simplified Procedure
1	SS steady	7.0	7.6
2	SS steady	7.4	7.6
3	SS crisis	8.9	9.5
4	SS crisis	9.6	9.3
5	SC crisis	11.3	11.4
6	SC crisis	14.4	14.2
7	SS crisis	13.8	13.1
8	SS crisis	13.9	12.6
9	Thalassaemia	33.4	34.8
10	Thalassaemia	35.3	34.8
11	Thalassaemia	64.5	63.9
12	Thalassaemia	67.5	66.0
	Mean ± SE	23.92 ± 6.28	23.73 ± 6.19

Blood samples were assayed for %F^+cells in parallel using the original and simplified procedures. There was no significant difference in the results between the 2 procedures.

3.2.5. Anticoagulants and sample stability for F^+cell assay

Study design

In order to search for suitable anticoagulants and sample stability for the simplified procedure, the effect of different anticoagulants and duration of storage at 4°C was examined.

Blood samples from a healthy volunteer (containing 0.6% HbF) and a sickle patient receiving hydroxyurea treatment (containing 17.6% HbF) were taken into EDTA, citrate or heparin anticoagulants. The samples were assayed for $\%F^+$cells using the simplified procedure (see section 3.2.3) on the day of collection (day 1) then stored at 4°C and re-assayed on days 2, 4, 10 and 15 respectively.

Results and discussion

The $\%F^+$cells of the normal sample on day 1, 2, 4, 10 and 15 in EDTA were 2.80, 2.78, 2.75, 2.73 and 2.75% respectively; in citrate were 2.73, 2.75, 2.72, 2.74 and 2.73% respectively and in heparin were 2.69, 2.70, 2.71, 2.70 and 2.68% respectively (Figure 3.2.5.1). These values of the sickle cell sample in EDTA were 57.30, 60.95, 60.61, 63.05 and 63.72% respectively; in citrate were 56.06, 60.48, 59.06, 58.27 and 63.58% respectively and in heparin were 59.36, 61.74, 56.82, 60.33 and 61.79% respectively. There was no significant difference between them (Figure 3.2.5.2).

These findings indicate that EDTA, citrate and heparin are all suitable for the F^+cell assay using single-colour staining flow cytometry and the sample can be kept at 4°C for up to 15 days prior to fixation and staining without affecting F^+cell results. EDTA was chosen as it is the most convenient one, because blood remaining from a routine FBC could be used.

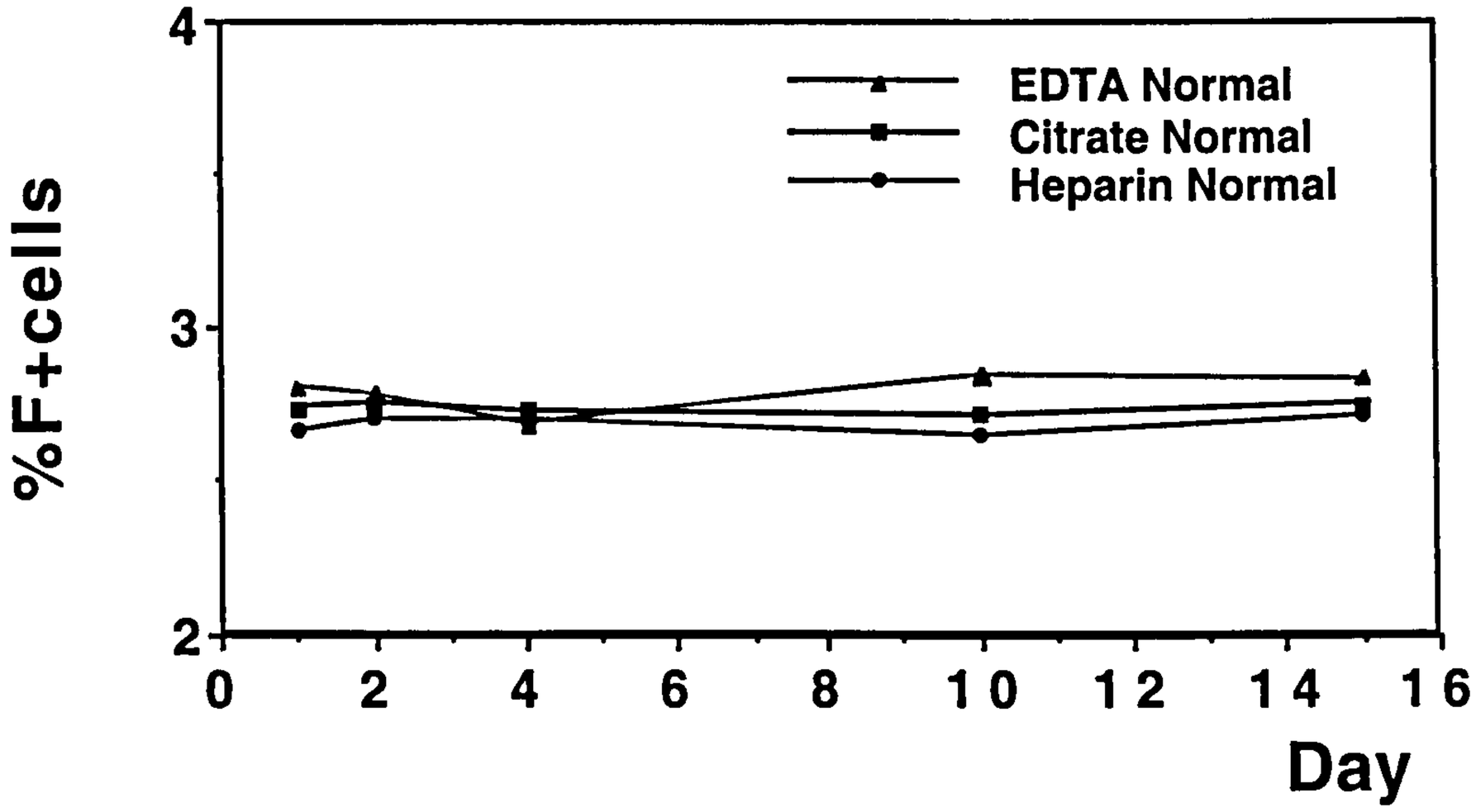


Figure 3.2.5.1 Effect of anticoagulants and stability of a normal blood sample

A normal blood sample (0.6 %HbF) was taken into EDTA, citrate and heparin and then assayed for $\%F^{+}$cells on days 1 (the day of collection), 2, 4, 10 and 15. The samples were kept at 4°C during the study period.

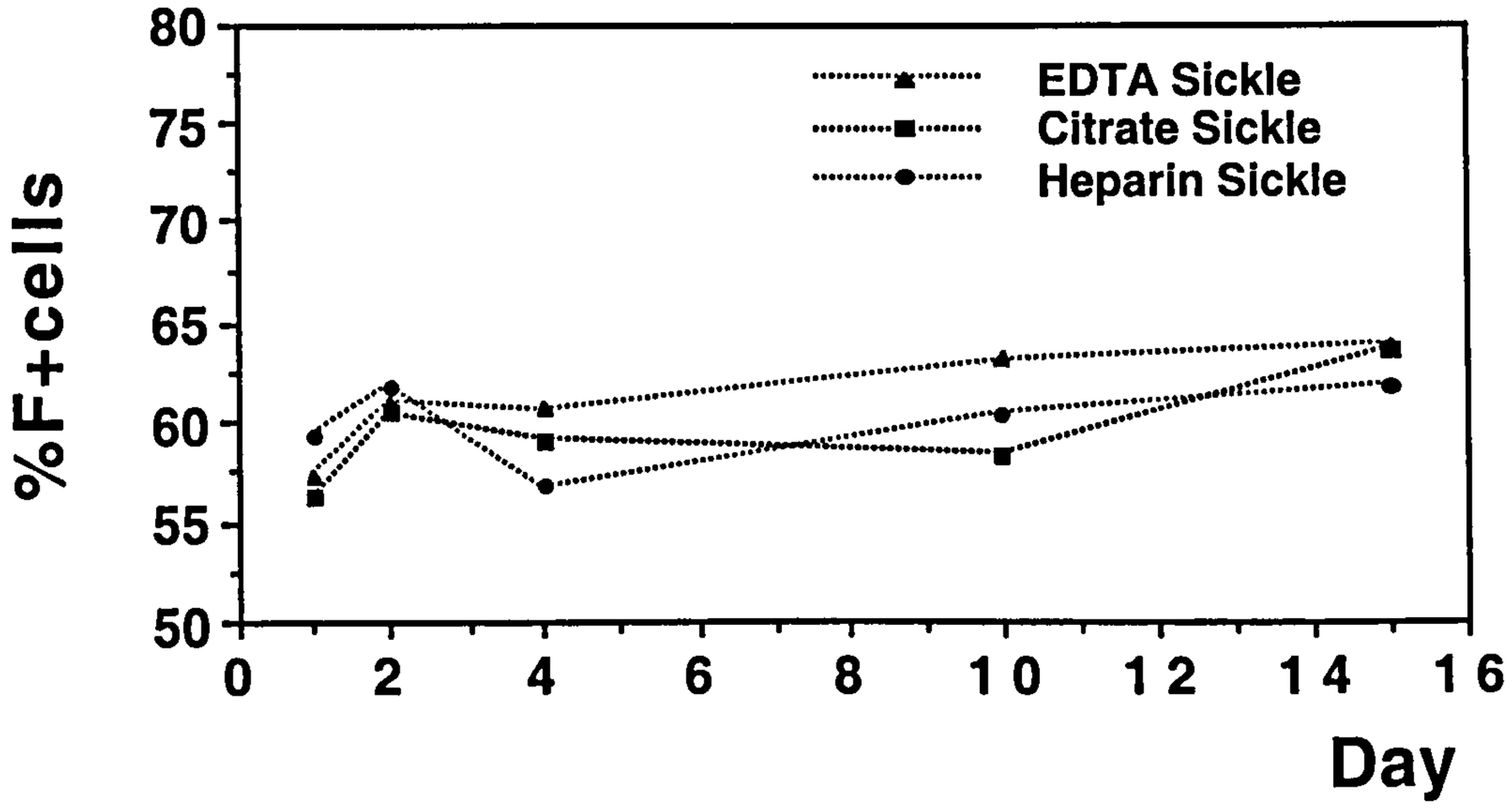


Figure 3.2.5.2 Effect of anticoagulants and stability of a sickle blood sample

A sickle blood sample (17.6 %HbF) was taken into EDTA, citrate and heparin and then assayed for $\%F^{+}$cells on days 1 (the day of collection), 2, 4, 10 and 15. The samples were kept at 4°C during the study period.

3.2.6. Reproducibility of simplified F^+cell assay

Study design

The reproducibility of an assay can be estimated by the intra- and inter-assay percent coefficient of variation (%CV). In general, an intra-assay %CV ≤10% and an inter-assay %CV ≤20% are regarded as acceptable for most assays. The lower %CV is the better reproducibility. EDTA was used as the most convenient anticoagulant.

EDTA blood samples were taken from sickle cell patients and normal healthy blood donors. Full blood count (FBC) with reticulocyte count was performed on the Sysmex SE-9500. Haemoglobin typing and quantitation was performed on the BioRad HPLC. Samples were centrifuged, the plasma was removed, and an equal volume of Alsever's solution : PBS (1:1) mixture was added to the packed red cells to obtain an approximately 45-50 % haematocrit. The samples were then stored at 4°C prior to assay.

The $\%F^+$cells was assayed on three blood samples of low, medium and high HbF levels (2.1, 5.6 and 9.4% respectively) using the simplified procedure (see section 3.2.3). Five replicates of intra-assay of each sample were obtained on the day of collection. The samples were then kept at 4°C. Five replicates of inter-assay of each sample were obtained by performing a replicate daily for a further 4 consecutive days. The inter- and intra-assay %CVs were determined.

Results and discussion

The intra-assay %CVs of blood samples containing high, medium and low %HbF were 1.36, 1.54 and 2.52% respectively, and the inter-assay %CVs were 2.67, 3.06 and 6.59% respectively (Table 3.2.6).

Table 3.2.6 Percentages of intra- and inter-assay coefficient of variation

%F^+cells	Mean	SD	%CV	Replicates
Intra-assay				
Low	11.50	0.29	2.52	5
Medium	25.26	0.39	1.54	5
High	42.42	0.58	1.36	5
Inter-assay				
Low	13.65	0.90	6.59	5
Medium	23.20	0.71	3.06	5
High	41.48	1.11	2.67	5

Three EDTA blood samples of low, medium and high HbF levels (2.1, 5.6 and 9.4% respectively) were used for determination of the inter- and intra-assay %CVs. The intra-assay data were obtained on the first day of collection for 5 replicates. The inter-assay data were obtained for another 4 consecutive days. The %CVs of the intra- and inter-assay fell within an acceptable range.

3.2.7. Relationship between %HbF and %F^+cells

Study design

A total of 70 blood samples, regardless of diagnosis were selected to include various percentages of HbF, and assayed for both %HbF using HPLC (sections 2.4.2) and %F^+cells using the simplified flow cytometric procedure (section 3.2.3). Samples included 25 normal control subjects, 37 sickle cell and 8 thalassaemic patients. The correlation coefficient (r) between these two parameters was then determined.

Results and discussion

The correlation coefficient (r) between %HbF and %F^+cells was 0.968 (Figure 3.2.7). The correlation was weaker when HbF was ≤1% (r=0.730, n=20) compared to the correlation when HbF was in between 1-10% (r=0.924, n=42) or >10% (r=0.894, n=8) respectively.

The best correlation was found in the range of 1-10%HbF. However, the correlation was still good when the HbF was less than 1% and greater than 10%. The lowest detectable level of $\%F^+$cells was 0.3%, because the cut-off point on the flow cytometer was set at this level. The %HbF detection of BioRad HPLC at levels under 1% is not accurate (data from BioRad Variant instruction manual), where the detection limit lies between 0.5 and 1%. However, %HbF and $\%F^+$cells are not exactly the same. %HbF is the proportion of HbF in the total Hb in a haemolysate, while $\%F^+$cells is the proportion of HbF containing red cells in the total red cells. Any red cells that contain sufficient amounts of HbF to trigger the fluorescent detector will be counted as F^+cells. Whereas in a haemolysate, a small amount of HbF may not be detected.

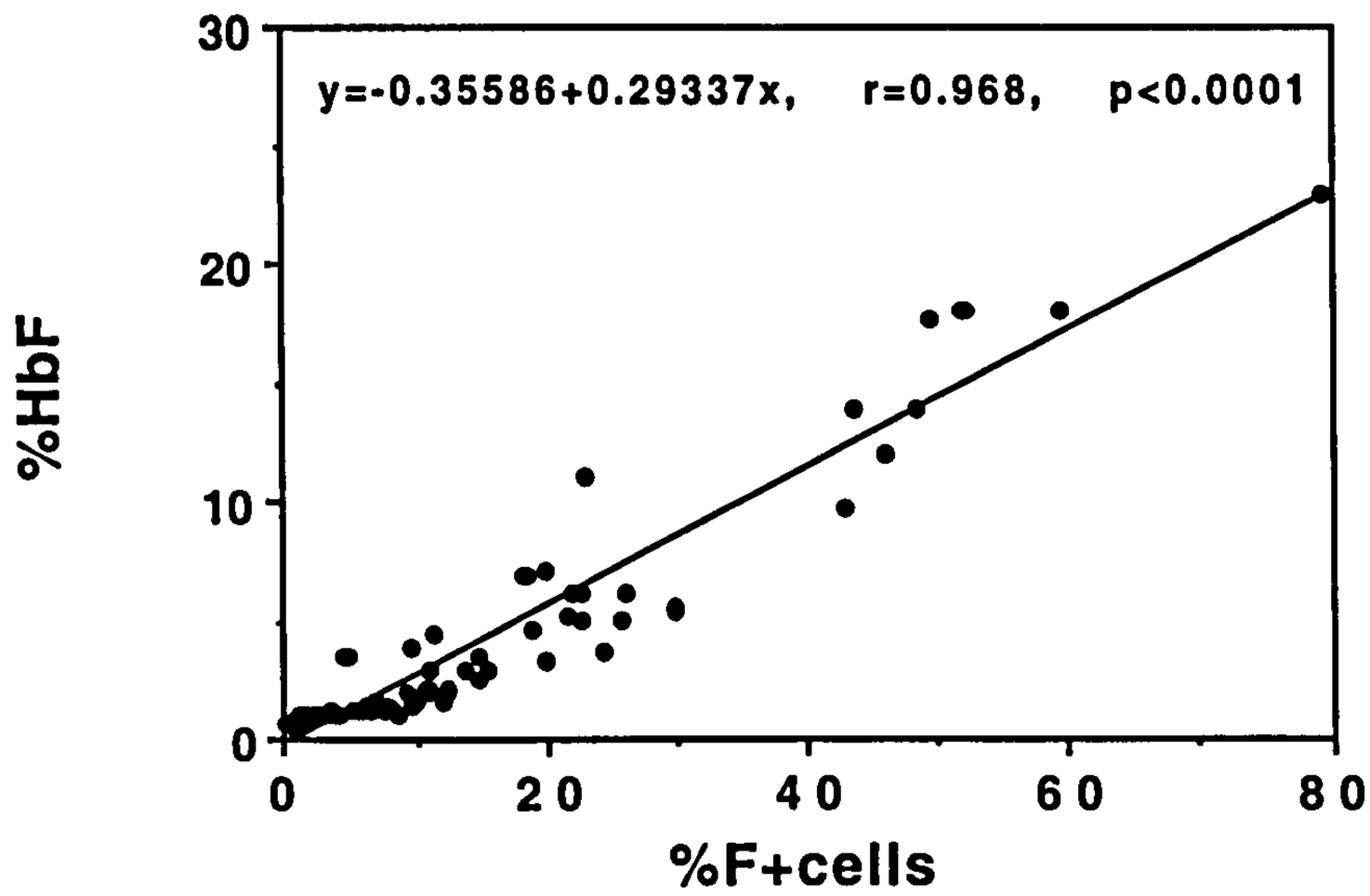


Figure 3.2.7 Relationship of %HbF and $\%F^+$cells

The %HbF (measured by HPLC) and $\%F^+$cells (measured by the simplified procedure) were obtained from 70 blood samples with different levels of HbF regardless of diagnosis, including 25 normal donors, 37 sickle cell patients and 8 thalassaemic patients. The overall coefficient of correlation (r) was 0.968 (n=70). When %HbF was ≤1% the r was 0.730 (n=20), when %HbF was between 1-10% the r was 0.924 (n=42) and when %HbF was >10% the r was 0.894 (n=8).

Furthermore, the concentration of HbF in an F^+cell may vary from 5 to 40% of total Hb within the cell. As a result, %HbF and %F^+cells in the same individual are not regularly correlated but should be approximately correlated. For example, only 30% HbF may produce almost 100% F^+cells, because every cell contains a certain amount of HbF which is high enough for the cell to be counted as a F^+cell. On the other hand, 30% HbF may produce only 60% F^+cells because the other 40% of cells contain very low levels of HbF which are not enough to trigger the fluorescent detector. However in practice, %F^+cells correlated well with %HbF.

In conclusion, the simplified flow cytometry procedure for %F^+cell assay correlated well with %HbF quantitation by HPLC. This finding suggests that assay of the %F^+cells by flow cytometry may be useful if quantitative assay of %HbF is not available in the laboratory.

3.2.8. Relationship between HbF and F^+cells in different groups of subjects

In this section, the relationship between %HbF and %F^+cells in different groups of subjects was investigated, whether the relationship was different for patients with different diagnoses.

Study design

The correlation coefficient (r) of the different groups of patients was determined and compared using data from section 3.2.7. Seventy blood samples included 25 normal controls, 18 sickle cell patients in steady state (SS.std.), 12 sickle cell patients in crisis (SS.cri.), 7 sickle cell patients receiving hydroxyurea treatment (SS.HU.) and 8 thalassaemic patients (Thal.int.).

Results

The correlation coefficient (r) was 0.863 in control group, 0.943 in SS.std. group, and 0.948 in SS.cri. group, 0.947 in SS.HU. group and 0.682 in Thal.int. group respectively. The correlation between these two parameters was highly significant ($p<0.001$) in all groups.

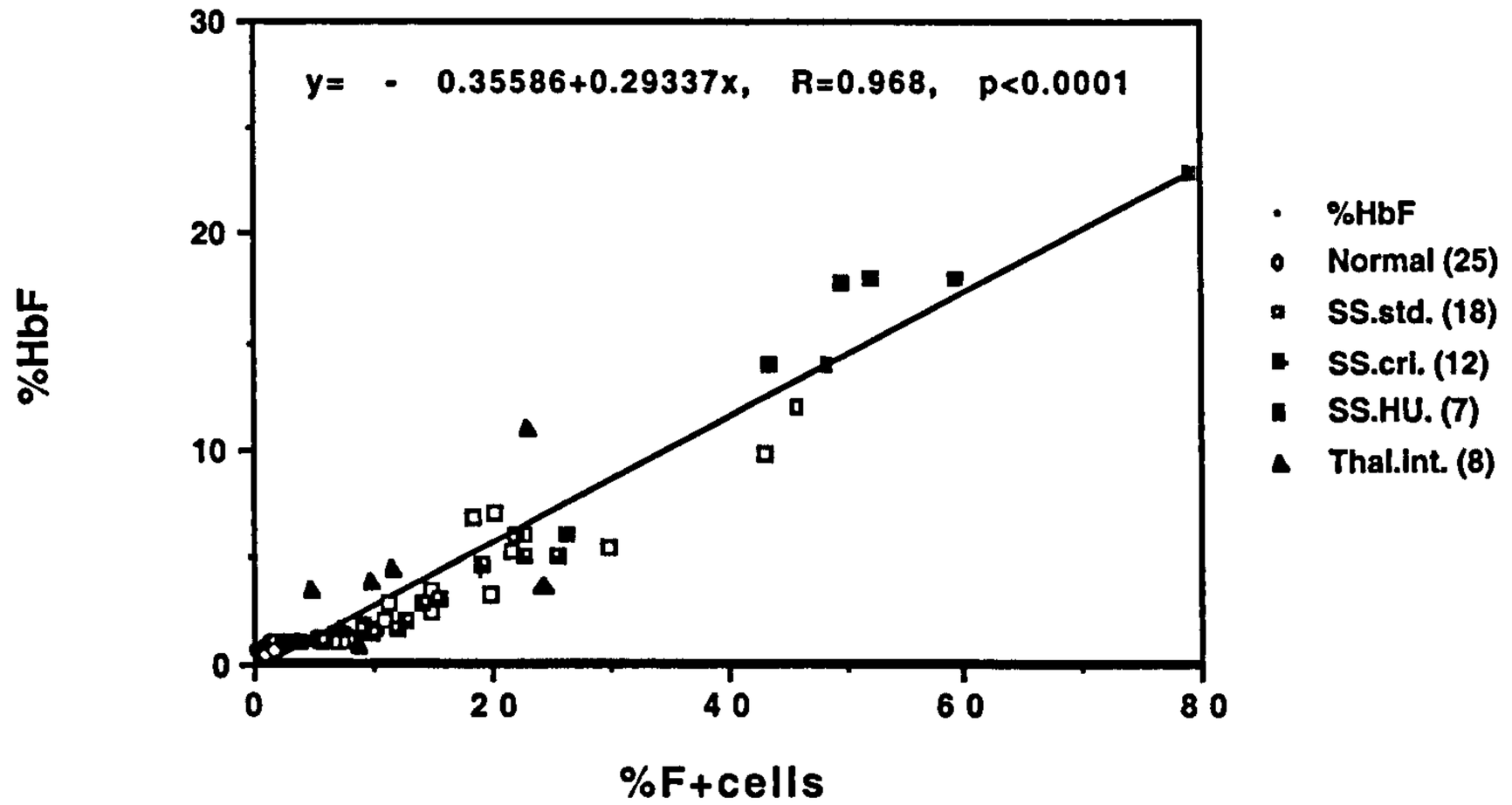


Figure 3.2.8 Relationship of %HbF and %F$^+$cells in different group of patients

The %HbF (measured by HPLC) and %F$^+$cells (measured by the simplified procedure) were obtained from 70 blood samples. The correlation between these two parameters was highly significant ($p<0.001$) in all groups.

Discussion

In every group of subjects, the %F$^+$cells were correlated with %HbF. Any individual, regardless of diagnosis, who had increased levels of HbF also had increased in F$^+$cells. This probably indicates that the amount of HbF per F$^+$cells is possibly nearly constant. However, in the normal and the thalassaemic group, the two parameters seem to show lesser correlation.

3.2.9. Comparison of FITC and Tri-color fluorochromes for F^+cell assay

Study design

Monoclonal antibody (MoAb) to human HbF conjugated to two different fluorescent dyes, fluorescein isothiocyanate (FITC) and tri-color (TC). are available. Either conjugate can be used in single-colour staining for flow cytometry. The results of $\%F^+$cells obtained using these two different dyes should not be significantly different, since the antibody is the same. In order to ensure this, a comparison between using FITC and TC conjugates was performed.

A total of 90 blood samples, regardless of diagnosis with various levels of HbF, were assayed using the simplified procedure (section 3.2.3). MoAb-HbF-FITC and MoAb-HbF-TC conjugates were used in parallel. The samples were collected from 17 control subjects, 20 thalassaemic and 53 sickle cell patients. The $\%F^+$cells obtained from these two conjugates were dot-plotted against each other and the coefficient of correlation (r) was determined. The mean values of the two groups of $\%F^+$cells obtained from using these 2 dyes were compared.

Results and discussion

The mean $\%F^+$cells obtained from using MoAb-HbF-FITC and MoAb-HbF-TC were 22.13$\pm$1.83 and 19.13$\pm$1.69 respectively with no significant difference. The coefficient of correlation (r) between these two dyes was 0.986 (Figure 3.2.9).

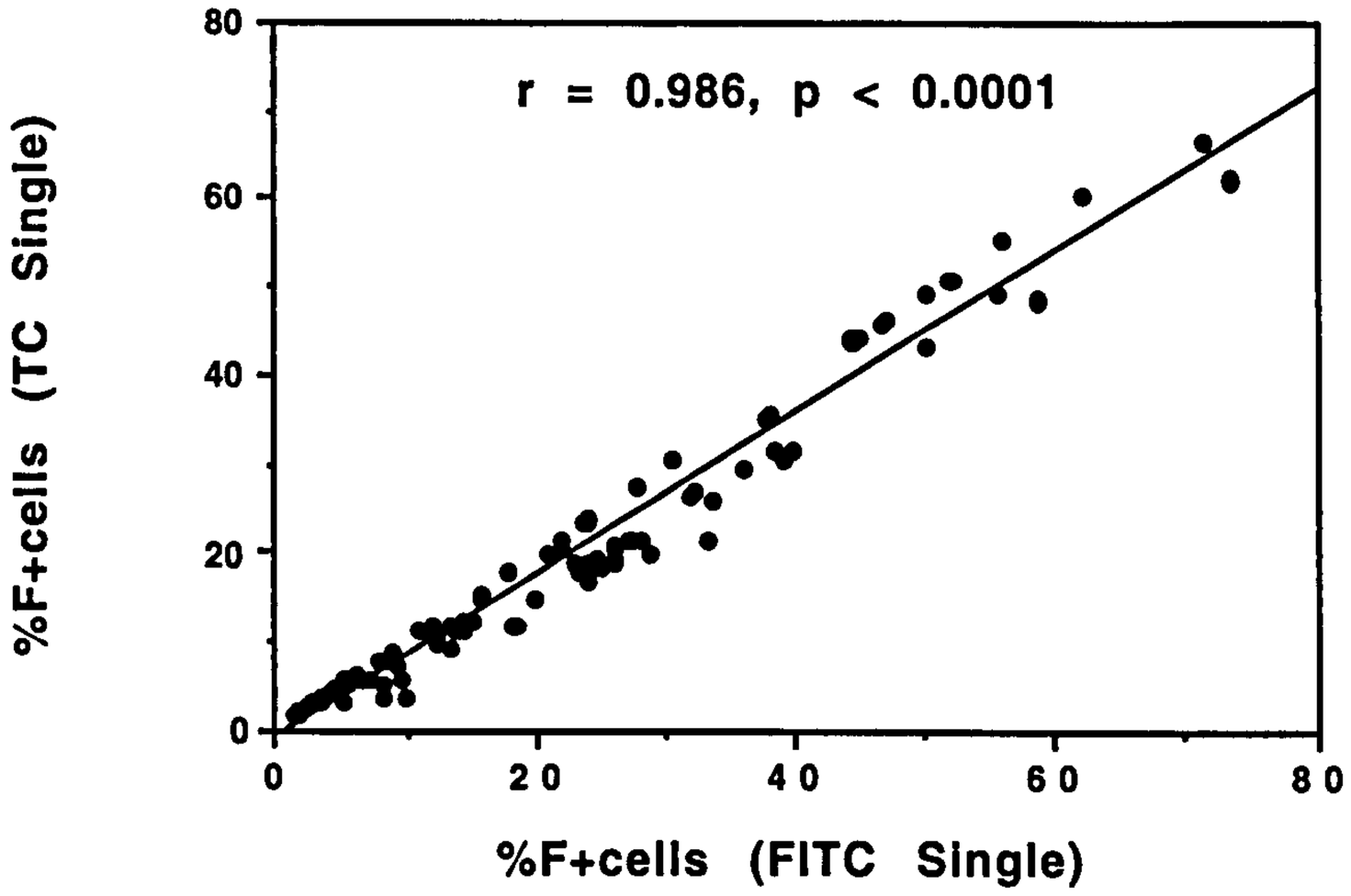


Figure 3.2.9 %F⁺cells obtained by using FITC and TC conjugates

Ninety blood samples regardless of diagnosis were assayed in parallel for %F⁺cells by the simplified procedure using either anti-HbF FITC or TC conjugate. They were collected from 17 normal donors, 20 thalassaemic patients and 53 sickle cell patients to include various levels of HbF. There was no significant difference between overall mean values of %F⁺cells obtained from using these two dyes. The coefficient of correlation (r) was 0.986 (n=90).

A good correlation was found between %F⁺cells obtained from using MoAb-HbF-FITC and MoAb-HbF-TC, and the overall mean values were not significantly different. However, the results obtained using FITC conjugate appeared slightly higher, probably because of the slightly higher intensity of the FITC signal. These findings show that either FITC or TC conjugate can be used without any significant effect on the results obtained.

3.2.10. Reticulocyte assay using single-colour staining flow cytometry

Thiazole orange (TO) is an RNA staining dye. It is excited at 509 nm then emits green fluorescence at 533 nm, which can be detected by FL1 on the Coulter Epics XL-MCL flow cytometer. TO easily penetrates the red cell membrane to stain RNA inside the cytoplasm of reticulocytes. It can be used as a single-colour stain for %reticulocyte assay by flow cytometry. The single-colour

staining flow cytometry for reticulocyte assay was used for comparison with either double- or triple-colour staining later. The method of staining are shown below.

Twenty μl of whole blood, pre-adjusted to 45-50% haematocrit by a mixture of Alsever's solution and PBS at the ratio 1:1, was diluted with 1 ml 0.1%BSA-PBS, then 100 μl of this diluted sample was further diluted with 900 μl of 0.1%BSA-PBS (final dilution is approximately 1 in 510). Twenty μl of final diluted sample was stained for RNA by adding 500 μl of 100 ng/ml Thiazole orange (TO) reagent (green) and incubating in the dark at RT for 30 min. This suspension was then analysed by flow cytometry within 3.5 hr. Since the fluorescent signal gradually fades. Non-stained control cells were prepared in the same way but at the last step 500 μl PBS was used instead of TO reagent.

3.2.11. Adhesion molecule expressing cell assay using single-colour staining flow cytometry

The single-colour staining flow cytometry for AM expressing cell (AM^+cell) assay was used for comparison with either double- or triple-colour staining later. The monoclonal antibodies against each AM were used together with anti-mouse-globulin conjugated to rhodamine phycoerythrin (RPE). The method of staining are shown below.

Twenty μl of whole blood, pre-adjusted to 45-50% haematocrit by a mixture of Alsever's solution and PBS at the ratio 1:1, was diluted with 1 ml 0.1%BSA-PBS, then 100 μl of this diluted sample was further diluted with 900 μl of 0.1%BSA-PBS (final dilution is approximately 1 in 510). Twenty μl of the final dilution was separately incubated with 20 μl, 1 in 5 diluted monoclonal antibody to each adhesion molecule (MoAb to CD36, CD41 and CD49d) at RT for 2 hr, then washed twice with 0.1%BSA-PBS. One set of three tubes of the above sample (one tube for each AM or three tubes together for three AMs) was incubated with 10 μl of 1 in 10 diluted anti-mouse-globulin conjugated to rhodamine phycoerythrin (RPE, red) in the dark at RT for 2 hr, and then washed once. The samples were then diluted with 500 μl 0.1%BSA-PBS and were ready

to be analysed by flow cytometry. Isotype controls were used instead of MoAb-AMs as a negative controls for non-specific binding.

3.3. DEVELOPMENT AND VALIDATION OF DOUBLE-COLOUR STAINING

3.3.1. Double-colour staining for F^+reticulocytes by flow cytometry

Rationale

Having evaluated single colour staining, the method was developed further with the aim of identifying a simple flow cytometric assay for F^+reticulocyte determination. The challenge was to choose a method where the fluorochrome used for RNA staining did not interfere with the fluorochrome used for detection of HbF. It was also important that the red cells remained stable using the two colour analysis and that use of double staining did not affect F^+cell assay.

Method

Blood samples were adjusted to approximately 45-50% Hct using the mixture of Alsever:PBS (1:1), then 20 µl was fixed with 1 ml ice-cold 0.05% glutaraldehyde in PBS at RT for 10 min. One hundred µl of fixed red cell suspension was permeabilised with 400 µl ice-cold 0.1% Triton X-100 in 0.1%BSA-PBS for 5 min (final dilution is approximately 1 in 255). Ten µl of fixed/permeabilised red cell suspension was then incubated with 20 µl of 1 in 10 diluted MoAb-HbF-TriColor (TC) and 70 µl 0.1%BSA-PBS in the dark at RT for 30 min. The total stained red cell suspension was then incubated with 500 µl Thiazole orange reagent (TO), in the dark at RT for 30 min. This suspension was analysed by flow cytometry within 3.5 hours (waiting longer than that the green fluorescence of TO may fade). An IgG1 conjugated to TC (isotype control) was used instead of MoAb-HbF-TC and PBS was used instead of TO reagent as a negative control for non-specific binding. Representative histograms of double

staining for simultaneous assay of F^+cells, reticulocytes and F^+reticulocytes are shown in Figure 3.3.1.

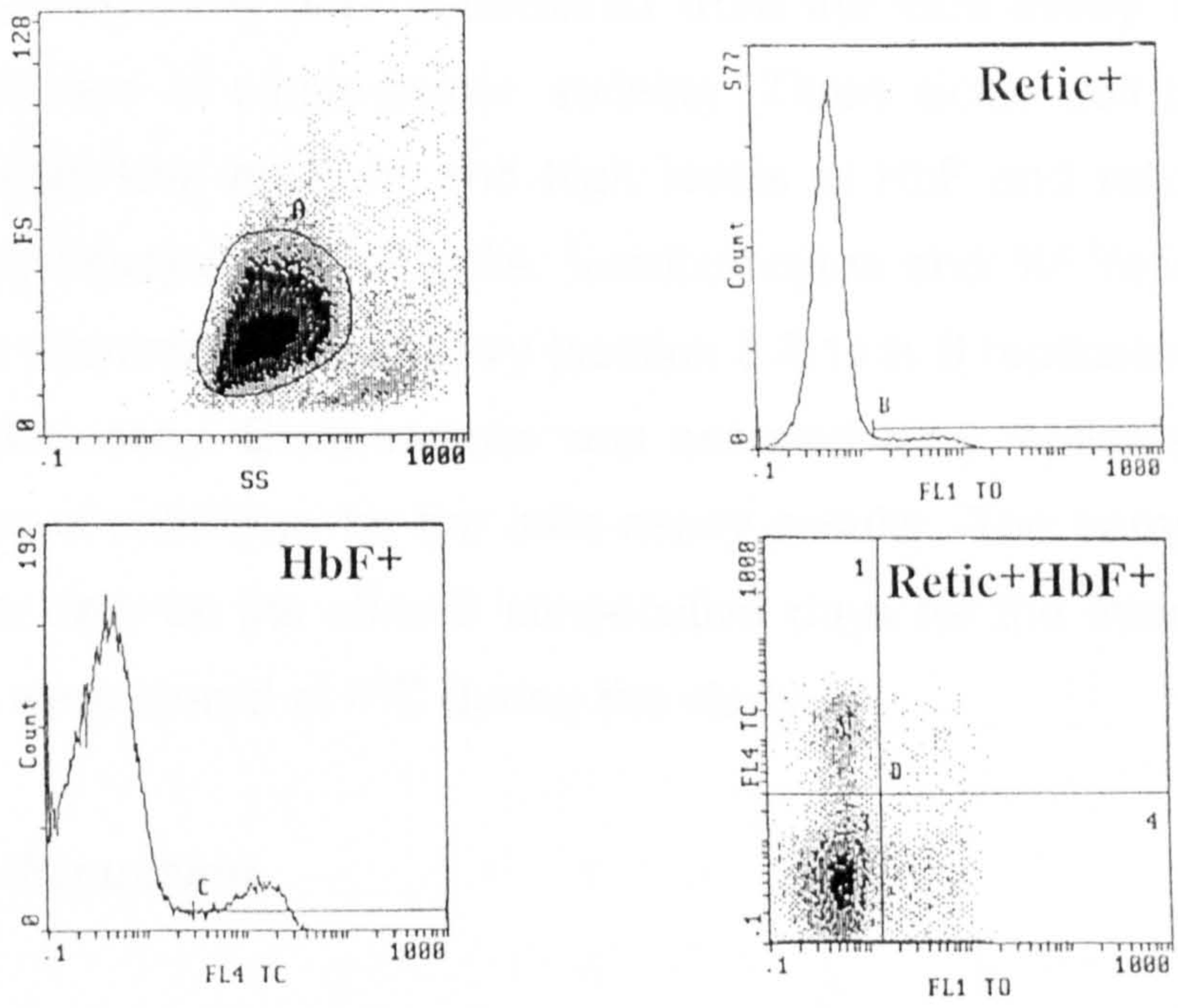


Figure 3.3.1 Flow cytometric histograms of double-colour staining

Top-left; This histogram shows red cell gate on a double channel (LogSS and FS) histogram.

Top-right; This histogram shows TO (green: LogFL1) signal on the horizontal axis against cell count on the vertical axis (for reticulocytes). The cut-off point separating the positive and negative population was set at 0.3% (+3SD) above the negative control population.

Bottom-left; This histogram shows TC (deep-red: LogFL4) signal on the horizontal axis against cell count on the vertical axis (for F^+cells). The cut-off point was also set at 0.3% above negative control population.

Bottom-right; This histogram shows a scatter plot of TO staining cells (reticulocytes) on the horizontal axis against TC staining cells (F^+cells) on the vertical axis (both on Log scale). The cut-off lines are obtained from the previous two histograms. The upper right quadrant represents the F^+reticulocyte (F^+R^+) area, the lower right quadrant represents the HbF negative reticulocyte (F^-R^+) area, the lower left quadrant represents the HbF negative mature red cell (F^-R^-) area and the upper left quadrant represents the F^+mature red cell (F^+R^-) area.

3.3.2. Reproducibility of double-colour staining

Study design

The reproducibility was determined from the intra-assay and inter-assay %CV as mentioned in single-colour staining. Three sickle cell blood samples, selected for their low, medium and high levels of HbF and reticulocytes, were simultaneously assayed for $\%F^+$cells, %reticulocytes and $\%F^+$reticulocytes using double-colour staining flow cytometry (section 3.3.1) in 6 replicates for both intra-assay and inter-assay. Each sample was assayed for 6 replicates in the same run on the day of collection for the intra-assay results. The samples were then assayed once daily on the other 5 consecutive days for the inter-assay results. The samples were stored at 4°C during the study.

Results and discussion

Table 3.3.2 Intra- and inter-assay percent coefficient of variation of double-colour staining flow cytometry

	Intra-assay			Inter-assay		
	$\%F^+$cells	%Retics	$\%F^+$retics	$\%F^+$cells	%Retics	$\%F^+$retics
Low	2.51	5.48	8.26	3.79	10.24	14.72
Med	1.40	4.33	7.83	2.90	10.00	12.14
High	1.19	2.52	6.30	2.21	7.42	8.36

Intra- and inter-assay %CVs fell within the acceptable range (not exceeding 10% and 20% respectively).

When double-colour staining for the simultaneous assay of F^+cells, reticulocytes and F^+reticulocytes was performed, the %CVs of all three parameters fell within the acceptable range of less than 10% for the intra-assay and less than 20% for the inter-assay. These findings demonstrated the good reproducibility of the method. Using of double-colour staining did not affect the reproducibility of the F^+cell assay.

3.3.3. Effect of anticoagulant and sample stability on double-colour staining

Study design

Blood samples from a normal subject and a sickle cell patient were collected in EDTA, citrate, heparin and CTAD (a special anticoagulant for platelet activation study). The samples were simultaneously assayed for %F^+cells, %reticulocytes and %F^+reticulocytes (section 3.2.3) on the day of collection (day 1) and for a further 5 consecutive days (day 2–6). The samples were stored at 4°C during the study. The mean results on each day were compared, in order to determine the effect of anticoagulants and sample stability.

Results and discussion

There was no significant difference between the results of %F^+cells, %reticulocytes or %F^+reticulocytes in normal and sickle blood samples taken into EDTA, citrate, heparin and CTAD. In addition, assaying every day up to 6 days on 4°C stored samples showed only a very small fall in the values obtained which did not reach statistical significance (Figure 3.3.3).

Any of the four anticoagulants used (EDTA, citrate, heparin and CTAD) appear suitable for the assay on samples stored at 4°C up to 5 days. These findings suggest that the EDTA samples from routine haematology laboratories, the citrate samples from coagulation laboratories or the heparin samples from special haematology laboratories are all suitable for the method, if they are stored at 4°C less than 5 days.

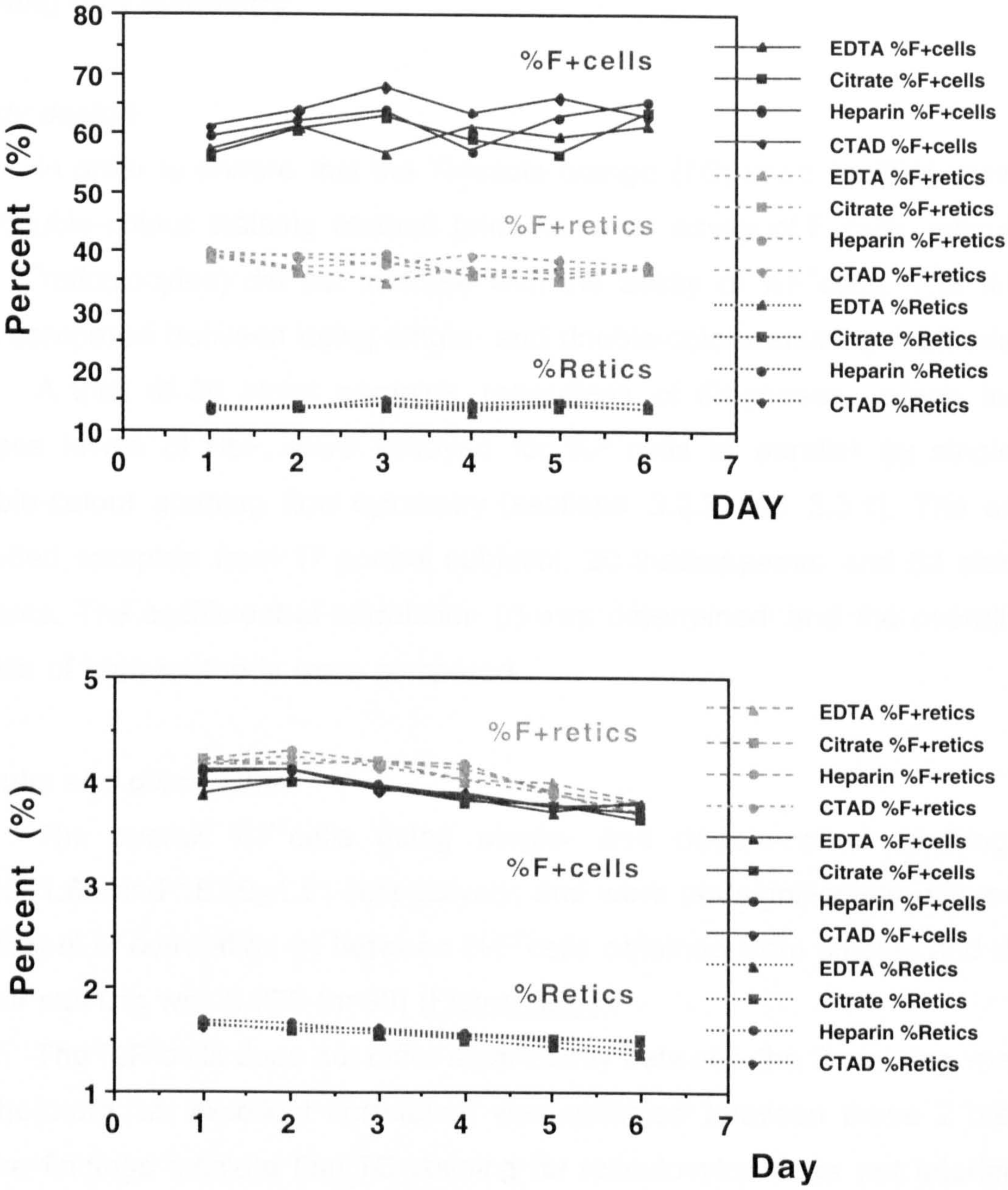


Figure 3.3.3 Effect of anticoagulants and sample stability

Blood samples from a sickle cell patient (top) and a normal subject (bottom) were collected into EDTA, citrate, heparin and CTAD. The plasma was removed and PBS:Alsever solution (1:1) was added for haematocrit of approximately 45-50%. The samples were assayed for %F^+cells, %reticulocytes, %F^+reticulocytes on the day of collection (day 1) and another five consecutive days (day 2–6). The samples were stored at 4°C during the study. There was no significant difference in these three parameters between the types of anticoagulant used or the length of 4°C storage up to 5 days.

3.3.4. Correlation between %F^+cells obtaining from single- and double-colour staining flow cytometry

Study design

In order to ensure that the Thiazole orange (TO) used for RNA staining in the double-colour staining method (simultaneous assay of F^+cells, reticulocytes and F^+reticulocytes) did not interfere with the assay of %F^+cells. The %F^+cells was compared between using single- and double-colour staining methods.

A total of 90 blood samples regardless of diagnoses, which included various levels of HbF, were assayed for %F^+cells in parallel by single- and double-colour staining flow cytometry (sections 3.2.3 and 3.3.1). The analysis included samples from 17 control subjects, 20 thalassaemic and 53 sickle cell patients. The coefficient of correlation (r) was determined and the overall mean results of both methods were compared.

Results and discussion

The overall %F^+cells using single- and double-colour staining were 19.13±1.69 and 18.59±1.61 respectively, and were not significantly different. The coefficient of correlation (r) between %F^+cells obtained from single- and double-colour staining was 0.996 (n=90) (Figure 3.3.4).

The %F^+cells does not differ significantly between the 2 staining methods. Furthermore, an excellent correlation was obtained between these 2 methods. These findings indicate that TO staining for reticulocytes does not interfere with the %F^+cell assay using TC in double-colour staining.

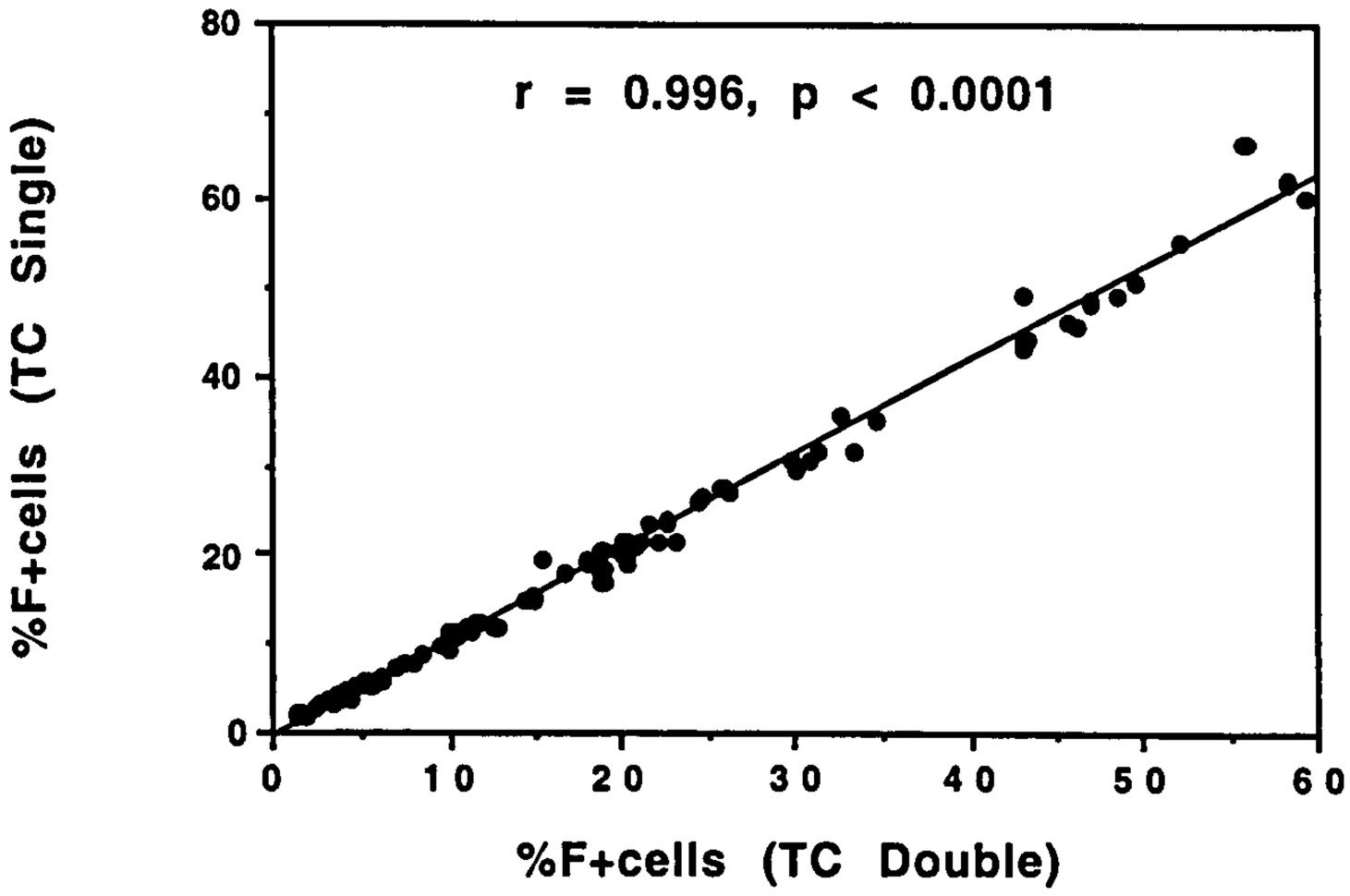


Figure 3.3.4 Comparison of %F$^+$cells obtained from single-colour and double-colour staining

90 blood samples, collected from 17 normal donors, 20 thalassaemic patients and 53 sickle cell patients were assayed for %F$^+$cells using single- and double-colour staining.

3.3.5. Correlation between %F$^+$cells obtained from double-colour staining flow cytometry and %HbF obtained from HPLC

Study design

From the above findings, it would be expected that %F$^+$cells using the double-colour method would correlate with %HbF by HPLC in a similar manner to results obtained with the single-colour method. A total of 90 blood samples regardless of diagnosis, which included various levels of HbF, were assayed for both %F$^+$cells using double-colour staining and %HbF using HPLC (sections 3.2.3 and 2.4.3 respectively). They were collected from 17 control subjects, 20 thalassaemic and 53 sickle cell patients. The coefficient of correlation (r) was determined and the overall mean results of both parameters were compared.

Results and discussion

The correlation coefficient (r) between %HbF and %F^+cells (double-colour staining) was 0.965 (n=90) (Figure 3.3.5). The overall mean %HbF was 4.87± 0.53 and the overall mean %F^+cells was 18.59±1.61.

The correlation between %F^+cells and %HbF is similar to the same type of correlation when %F^+cells was obtained from single-colour staining. As previously discussed, these two assays are different in principles but show a good correlation. The double-colour staining method does not differ in this aspect from single-colour staining.

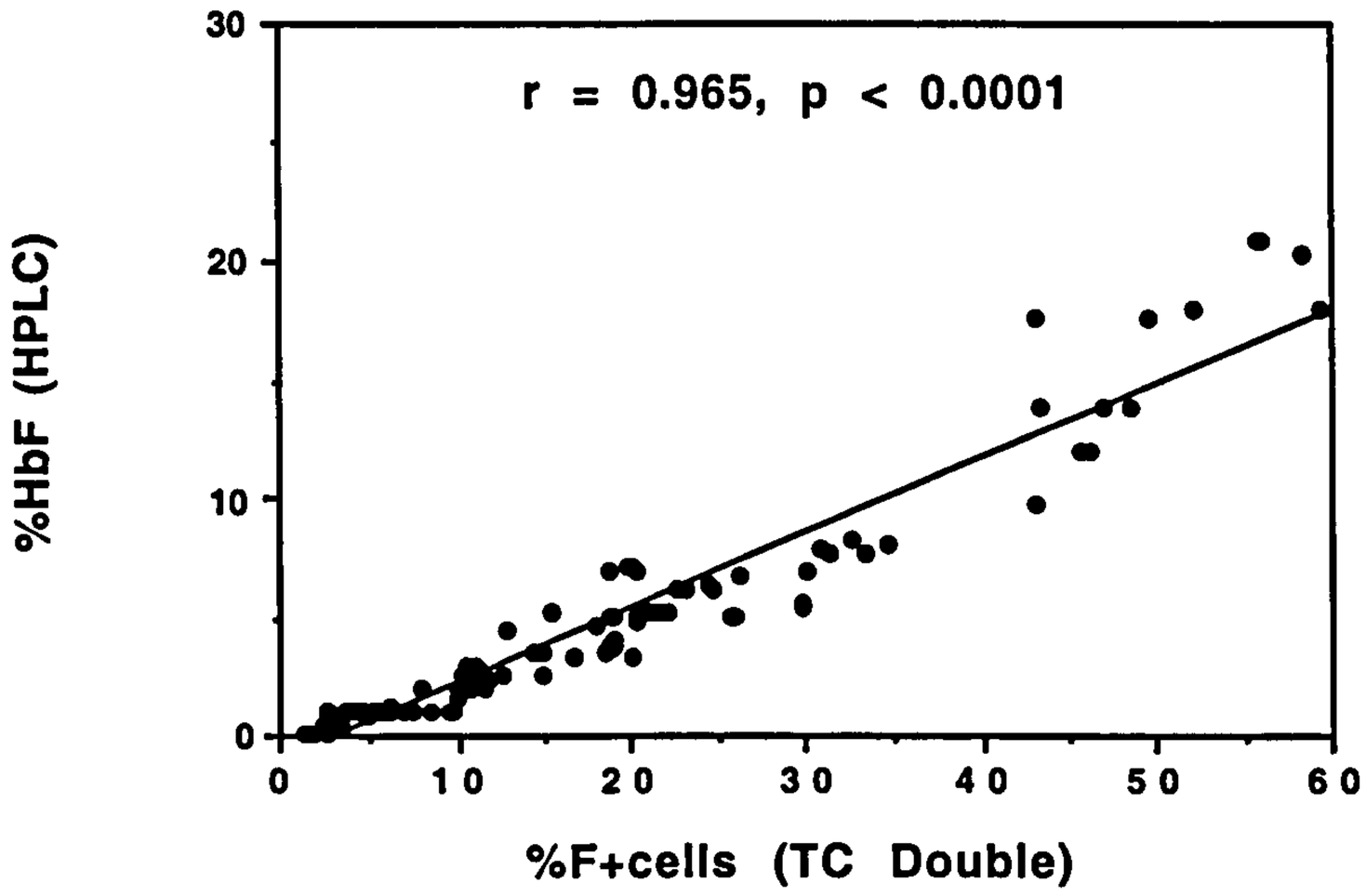


Figure 3.3.5 Relationship of %HbF and %F^+cells from double-colour staining

A total of 90 blood samples collected from 17 normal donors, 20 thalassaemic patients and 53 sickle cell patients were assayed for %HbF using HPLC and %F^+cells using double-colour staining flow cytometry for simultaneous assay of %F^+cells, %reticulocytes and %F^+reticulocytes.

3.3.6. Correlation of %reticulocytes obtained from single- and double-colour staining flow cytometry

Study design

It is important that the %reticulocytes obtained using the double-colour staining method is comparable to the value obtained from the single-colour staining. A total of 90 blood samples, regardless of diagnosis, which included various levels of reticulocytes, were assayed for %reticulocytes in parallel by single- and double-colour staining (sections 3.2.10 and 3.3.1). Samples were obtained from 17 control subjects, 20 thalassaemic and 53 sickle cell patients for this purpose. The coefficient of correlation (r) was determined and the overall mean results of both parameters were compared.

Results and discussion

The overall %reticulocytes obtained using single- and double-colour staining were 6.82±0.53 and 6.83±0.54 respectively. These values were not significantly different. The coefficient of correlation of %reticulocytes by single- and double-colour staining was 0.991 (n=90) (Figure 3.3.6).

A good correlation was obtained between the %reticulocytes determined by single- and double-colour staining and the overall mean values were not significantly different. These findings indicate that TC does not interfere with the assay of %reticulocytes using TO and *vice versa*.

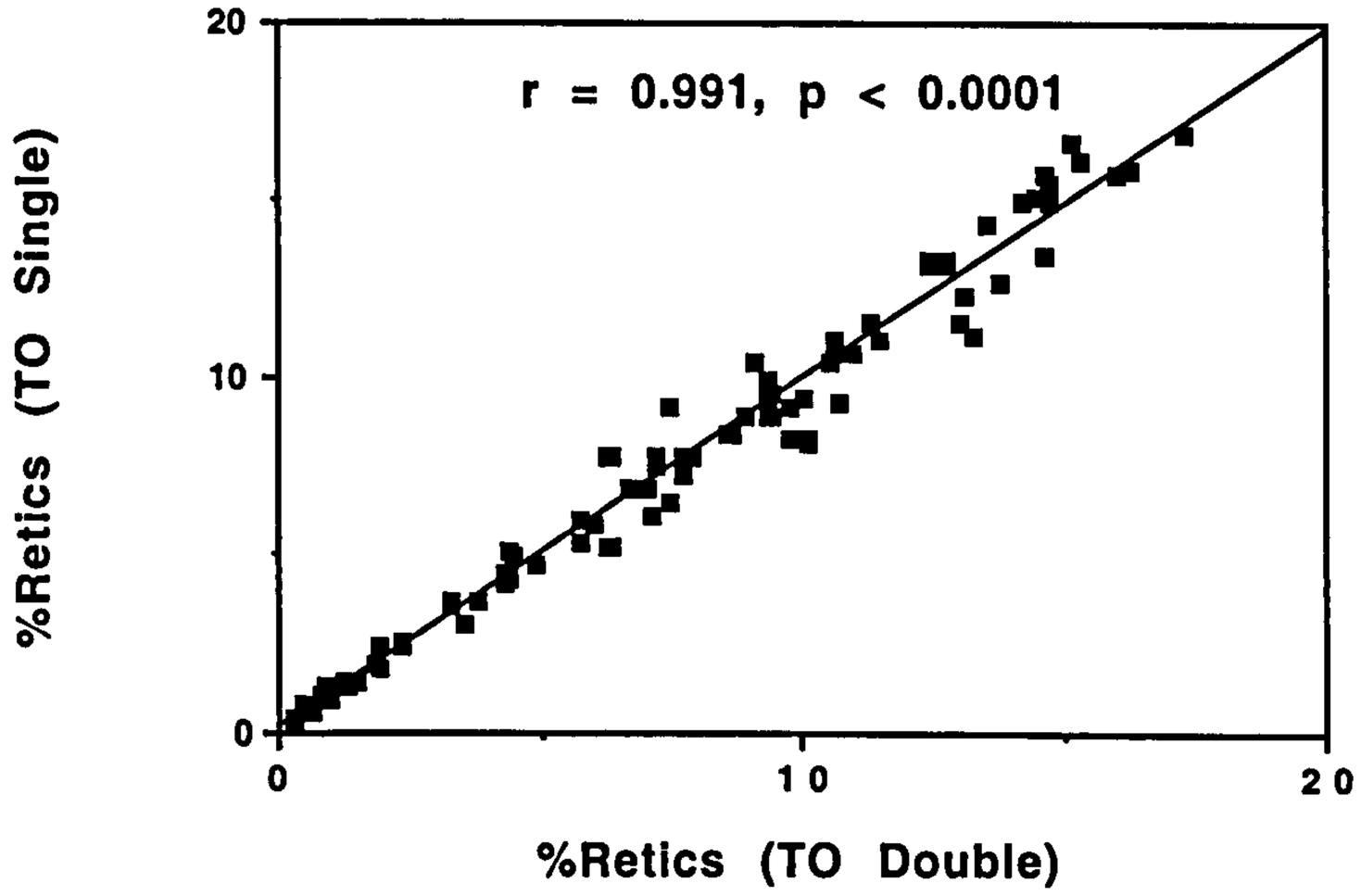


Figure 3.3.6 Comparison of %reticulocytes obtained from single-colour and double-colour staining

A total of 90 blood samples, collected from 17 normal donors, 20 thalassaemic patients and 53 sickle cell patients were assayed for %reticulocytes using single- and double-colour staining.

3.3.7. Correlation of %reticulocytes obtained from double-colour staining flow cytometry and Sysmex SE-9500 reticulocyte analyser

Rationale

Having established that double-colour staining for F^+reticulocytes did not affect the assay of reticulocytes by TO, a further experiment was performed to compare the %reticulocytes obtained using double-colour staining flow cytometry and using Sysmex SE-9500 reticulocyte analyser. There are many RNA stains, which can be used for the assay of reticulocytes. These include Thiazole orange (TO) and Auramine O. Sysmex SE-9500 uses Auramine O as the RNA dye and is available in routine haematology laboratory at UCLH. In order to ensure that the %reticulocytes obtained using the double-colour staining flow cytometry were comparable with a standard reticulocyte analyser, samples were assayed in parallel using both methods.

Study design

A total of 90 blood samples, regardless of diagnosis and including various levels of reticulocytes, were assayed for %reticulocytes in parallel by both Sysmex SE-9500 and double staining (section 3.3.1). Samples included 17 control subjects, 20 thalassaemic and 53 sickle cell patients. The coefficient of correlation (r) was determined and the overall mean results from both methods were compared.

Results and discussion

The overall %reticulocytes by the double-colour staining flow cytometry and Sysmex SE-9500 were 6.83±0.54 and 6.05±0.48 respectively, with non-significant difference between them. The coefficient of correlation of %reticulocytes obtained using this two methods was 0.974 (n=90) (Figure 3.3.7).

A good correlation was obtained between the %reticulocytes measured by double-colour staining and by Sysmex SE-9500 and the overall mean values were not significantly different. Slightly lower values were observed using the Sysmex SE-9500. This is possibly because TO staining give a slightly stronger signal than Auramine O used in Sysmex SE-9500.

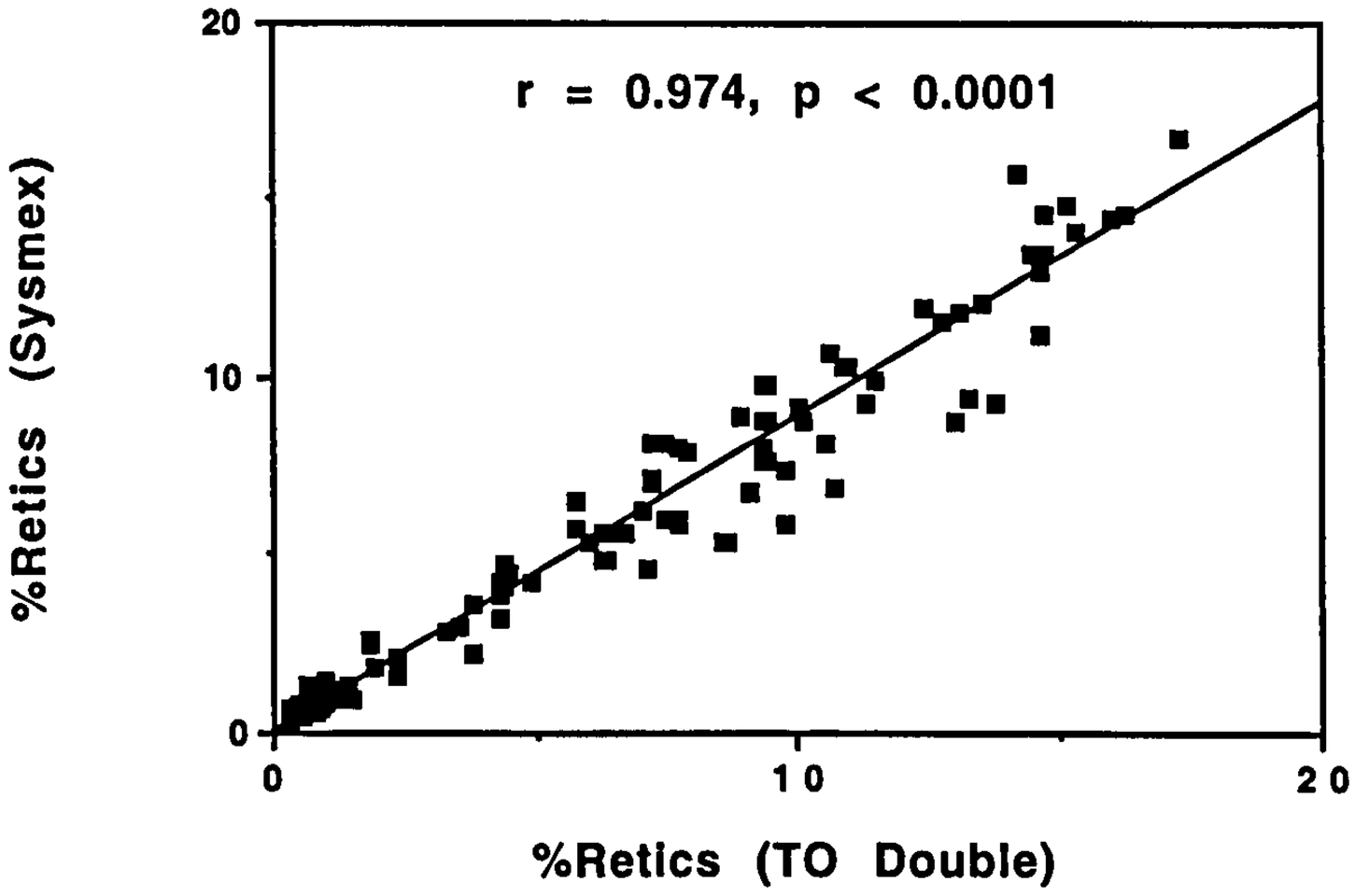


Figure 3.3.7 Comparison of %reticulocytes obtained from double-colour staining flow cytometry and Sysmex SE-9500 reticulocyte analyser

A total of 90 blood samples were collected from 17 normal donors, 20 thalassaemic patients and 53 sickle cell patients. %Reticulocytes obtained from the double-colour staining method for simultaneous assay of $\%F^+$cells, %reticulocytes and $\%F^+$reticulocytes were compared with the %reticulocytes obtained from the Sysmex SE-9500 reticulocyte analyser.

3.4. DEVELOPMENT AND VALIDATION OF TRIPLE-COLOUR STAINING

3.4.1. Background

A key goal in this thesis was to find a way of measuring red cell adhesion molecule expression on subpopulations of red cells. In particular, how to determine a co-distribution of AM expression, HbF content and RNA content of red cells. It was necessary therefore to develop a reliable surface staining for adhesion molecules on red cells while at the same time intracellular HbF and RNA can also be stained after permeabilisation. Thus the challenges were two fold; firstly to identify three fluorochromes which did not interfere with each other, and secondly to set up a method which allowed both surface and intra-

erythrocyte staining of the same red cells. Before going to triple-colour staining, three double-colour staining for the co-distribution of AMs on reticulocytes or on F^+cells was undertaken.

3.4.2. Three double-colour staining for co-distribution of RNA, AMs and HbF expressing cells

Thirty μl of packed red cells were diluted with 1 ml 0.1%BSA-PBS, then 100 μl of this diluted sample was further diluted with 900 μl of 0.1%BSA-PBS (final dilution approximately 1 in 340). Twenty μl of the final dilution was separately incubated with 20 μl 1 in 5 diluted monoclonal antibody to each adhesion molecule (AM-MoAbs to CD36, CD41 and CD49d) at RT for 2 hours (hr), then washed twice with 0.1%BSA-PBS. Three sets of reactions were prepared: one tube for simultaneous assay of F^+cells, reticulocyte and F^+reticulocytes, one set of 3 tubes for analysis of each AM expression on F^+cells and another set for the analysis of AM expression on reticulocytes.

For analysis of AM expression on F^+cells, one set of three tubes of the above samples (one tube for each AM or three tubes together for all three AMs) was fixed with 600 μl ice-cold 0.05% glutaraldehyde in PBS at RT for 10 min, washed once with 0.1%BSA-PBS and permeabilised with 600 μl ice-cold 0.05% Triton X-100 in 0.1%BSA-PBS for 10 min. After this the cells were washed once, then incubated with 10 μl 1 in 10 diluted anti-mouse-globulin conjugated to RPE (**red**) in the dark at RT for 2 hr and then washed twice. The cells were incubated with 10 μl 1 in 5 diluted MoAb-HbF-FITC (**green**) in the dark at RT for 30 min, washed once, and finally diluted with 600 μl 0.1%BSA-PBS. This suspension was then analysed by flow cytometry.

For analysis of AM expression on reticulocytes, one set of three tubes of the above samples (one tube for each AM or three tubes together for all three AMs) was incubated with 10 μl 1 in 10 diluted anti-mouse-globulin conjugate to RPE (**red**) in the dark at RT for 2 hr, washed once, then stained for RNA by adding 600 μl TO reagent (**green**) and incubating in the dark at RT for 30 min.

This suspension was then analysed by flow cytometry, which was carried out within 3.5 hr.

For simultaneous analysis of F^+cells, reticulocyte and F^+reticulocytes, one tube of 20 µl of the final dilution above was fixed with 600 µl ice-cold 0.05% glutaraldehyde in PBS at RT for 10 min and washed once with 0.1%BSA-PBS. Cells were permeabilised with 600 µl ice-cold 0.05% Triton X-100 in 0.1%BSA-PBS for 10 min, washed once and then incubated with 10 µl 1 in 5 diluted MoAb-HbF-TC (**deep-red**) in the dark at RT for 30 min and washed once. After this they were finally stained for RNA by adding 600 µl TO reagent (**green**) and incubating in the dark at RT for 30 min. This suspension was then analysed by flow cytometry, which was carried out within 3.5 hr.

Isotype controls were used instead of anti-AMs and anti-HbF and PBS was used instead of TO reagent as negative controls for non-specific binding. Representative histograms of the three double-colour staining for simultaneous assay of F^+cells, reticulocytes, F^+reticulocytes and adhesion molecule expression on F^+cells and on reticulocytes are shown in Figure 3.4.2.

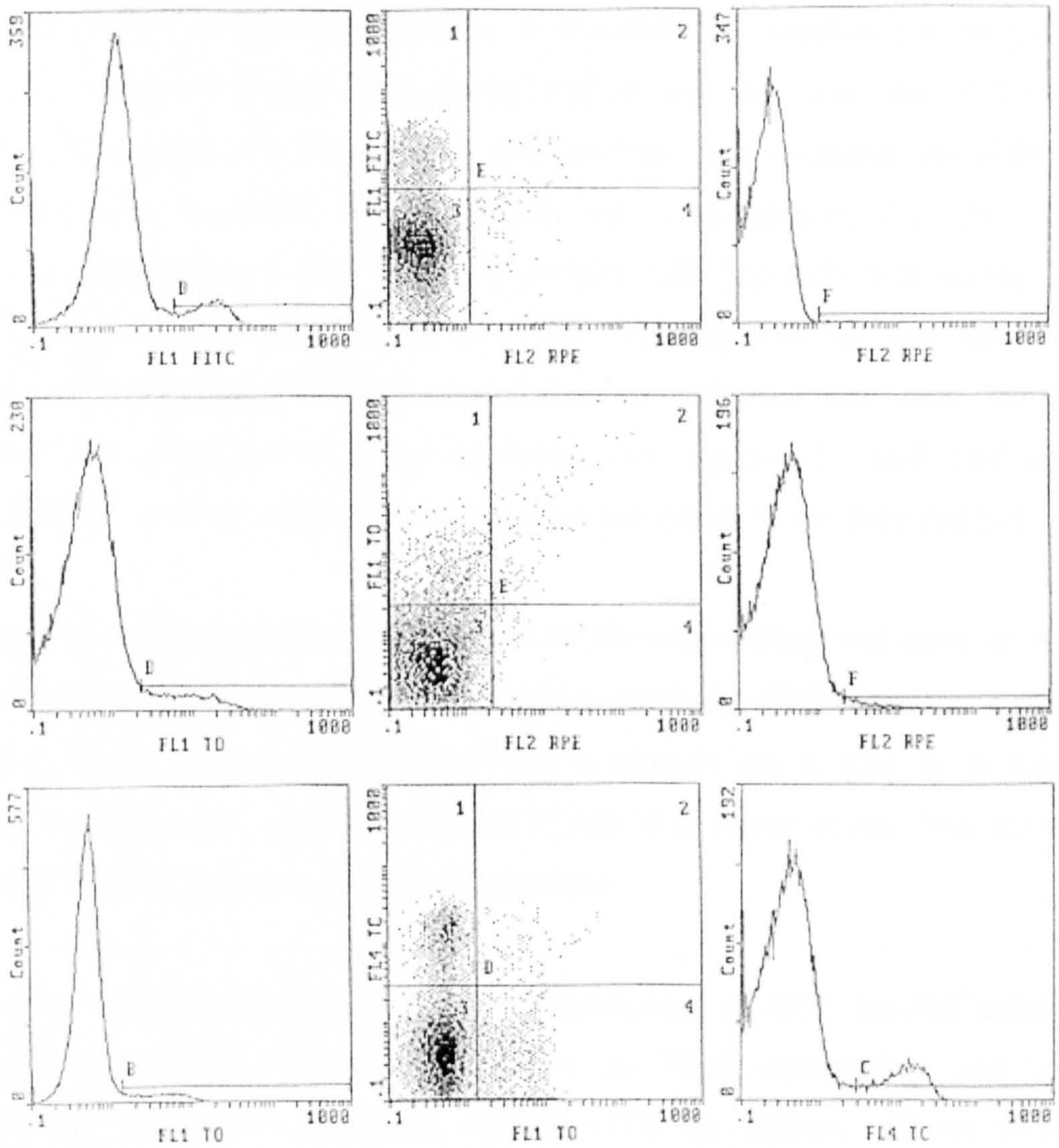


Figure 3.4.2 Flow cytometric histograms of three double-colour staining

Top row; Double staining of FITC (for F^+cells) *vs* RPE (for AM^+cells)

Left; A single channel histogram of HbF containing red cells or F^+cells, stained by MoAb-HbF-FITC is shown. Green fluorescent signal detected by FL1 is shown on X axis in a log scale. Number of cells (cell count) is shown on Y axis in a linear scale. The cut-off point was set at 0.3% above the negative population.

Middle; A double channel histogram of F^+cells and adhesion molecule expressing cells or AM^+cells is shown. Green fluorescent signal (FITC) detected by FL1 is shown on X axis in a log scale. Red fluorescent signal (RPE) detected

by FL2 is shown on Y axis also in a log scale. The horizontal cut-off line was set at 0.3% above the negative population, to separate the positive and negative HbF (FITC) or F^+cells and F^-cells. The vertical cut-off line was also set at 0.3% above the negative population, to separate the positive and negative AM (RPE) cells. These two lines separate the histogram into 4 quadrants (Q1-Q4). The cell number is presented in dots and dot density. **Q1** (top-left) represents ordinary F^+cells since they are positive for only HbF but negative for AM. **Q2** (top-right) represents AM^+F^+cells since they are positive for both HbF and AMs. **Q3** (bottom-left) represents ordinary red cells since they are negative for both HbF and AMs. **Q4** represents ordinary AM^+cells since they are positive for only AM but negative for HbF.

Right; A single channel histogram of AM expressing red cells or AM^+cells, stained by MoAb-AM coupled with anti-mouse-globulin-RPE conjugate is shown. Red fluorescent signal detected by FL2 is shown on X axis in a log scale. Number of cells (cell count) is shown on Y axis in a linear scale. The cut-off point was set at 0.3% above the negative population.

Middle row; Double staining of TO (for reticulocytes) *vs* RPE (for AM^+cells)

Left; A single channel histogram of RNA containing red cells or reticulocytes, stained by Thiazole orange (TO) be shown. Green fluorescent signal detected by FL1 is shown on X axis in a log scale. Number of cells (cell count) is shown on Y axis in a linear scale. The cut-off point was set at 0.3% above the negative population.

Middle; A double channel histogram of reticulocytes and AM^+cells is shown. Green fluorescent signal (TO) detected by FL1 is shown on Y axis in log scale. Red fluorescent signal (RPE) detected by FL2 is shown on X axis also in log scale. The horizontal cut-off line was set at 0.3% above the negative population, to separate the positive and negative RNA (TO) or reticulocytes and mature red cells. The vertical cut-off line was also set at 0.3% above the negative population, to separate the positive and negative AM (RPE) expressing cells. These two lines separate the histogram into 4 quadrants (Q1-Q4). The cell

number is presented in dots and dot density. **Q1** (top-left) represents ordinary reticulocytes since they are positive only for RNA but negative for AM. **Q2** (top-right) represents AM^+reticulocytes since they are positive for both RNA and AM. **Q3** (bottom-left) represents ordinary mature red cells since they are negative for both RNA and AM. **Q4** represents AM^+mature red cells since they are positive only for AM but negative for RNA.

Right; A single channel histogram of AM expressing red cells or AM^+cells, stained by MoAb-AM coupled with anti-mouse-globulin-RPE conjugate is shown. Red fluorescent signal detected by FL2 is shown on X axis in a log scale. Number of cells (cell count) is shown on Y axis in a linear scale. The cut-off point was set at 0.3% above the negative population.

Bottom row; Double staining of TO (for reticulocytes) *vs* TC (for F cells)

Left; A single channel histogram of RNA containing red cells or reticulocytes, stained by Thiazole orange (TO) be shown. Green fluorescent signal detected by FL1 is shown on X axis in a log scale. Number of cells (cell count) is shown on Y axis in a linear scale. The cut-off point was set at 0.3% above the negative population.

Middle; A double channel histogram of reticulocytes and F cells is shown. Green fluorescent signal (TO) detected by FL1 is shown on X axis in a log scale. Deep-red fluorescent signal (TC) detected by FL4 is shown on Y axis also in a log scale. The horizontal cut-off line was set at 0.3% above the negative population, to separate the positive and negative HbF (TC) or F^+cells and F^-cells (F negative cells or non-F cells). The vertical cut-off line was also set at 0.3% above the negative population, to separate the positive and negative RNA (TO) or reticulocytes and mature red cells. These two lines separate the histogram into 4 quadrants (Q1-Q4). The cell number is presented in dots and dot density. **Q1** (top-left) represents F^+mature red cells since they are positive only for HbF but negative for RNA. **Q2** (top-right) represents F^+reticulocytes since they are positive for both HbF and RNA. **Q3** (bottom-left) represents ordinary mature red cells

since they are negative for both HbF and RNA. **Q4** represents ordinary reticulocytes since they are positive for only RNA but negative for HbF.

Right; A single channel histogram of HbF containing red cells or F^+cells, stained by MoAb-HbF-TC is shown. The deep-red fluorescent signal detected by FL4 is shown on X axis in a log scale. Number of cells (cell count) is shown on Y axis in a linear scale. The cut-off point was set at 0.3% above the negative population.

3.4.3. Triple-colour staining flow cytometry for simultaneous assay of co-distribution of HbF, RNA and AMs

Rationale

Having shown the feasibility of simultaneous assay of AMs with either reticulocytes or F^+cells using three double-colour staining, a further method was devised for simultaneous analysis of all three variables on the same cell. The triple-colour staining method is developed and described below. In subsequent sections, validation of the stability of the sample preparation is shown.

Method

Thirty μl of packed red cells was diluted with 1 ml 0.1%BSA-PBS and 100 μl of this diluted sample was then further diluted with 900 μl 0.1%BSA-PBS (final dilution approximately 1 in 340). Twenty μl of the final dilution was separately incubated with 20 μl 1 in 5 diluted monoclonal antibody to each adhesion molecule (CD36, CD41 and CD49d, 1 tube each) at RT for 2 hr, then washed twice with 0.1%BSA-PBS. The suspension was incubated with 20 μl 1 in 10 diluted anti-mouse-globulin conjugated biotin at RT for 2 hr, then fixed with 600 μl ice-cold 0.05% glutaraldehyde in PBS for 10 min and washed once. This was then permeabilised with 600 μl ice-cold 0.05% Triton X-100 in 0.1%BSA-PBS for 10 min and washed once. After this the suspension was simultaneously incubated with 10 μl 1 in 5 diluted MoAb-HbF-TC (**deep-red**) and 10 μl 1 in 10 diluted streptavidin-RPE (**red**) in the dark at RT for 30 min and washed once. The

cell suspension was then incubated with 600 µl 100 ng/ml Thiazole Orange reagent (**green**) in the dark at RT for 30 min. This suspension was then ready for flow cytometric analysis within 3.5 hr. Isotype controls were used instead of anti-AMs and anti-HbF antibodies and PBS was used instead of TO reagent as negative controls for non-specific binding. The gate setting for triple-colour staining was described in chapter 2. Representative histograms of triple-colour staining for simultaneous assay of adhesion molecule expression on F^+cells, reticulocytes, and F^+reticulocytes are shown in Figure 3.4.3.

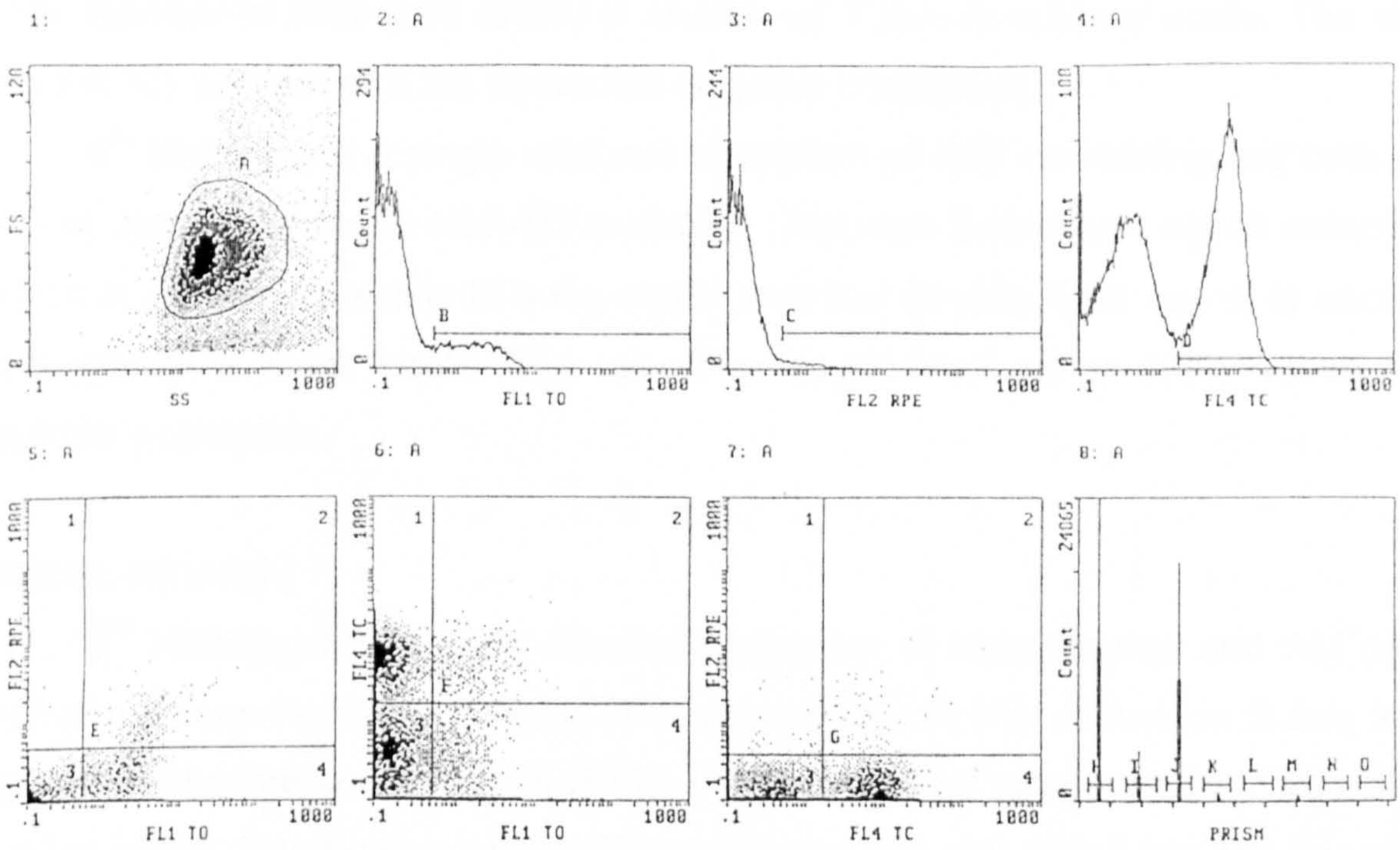


Figure 3.4.3 Flow cytometric histograms of triple-colour staining

Top, left→right

1st Histogram; A double-channel histogram for red cells is shown. The histogram shows forward scatter (FS) on the vertical axis in a linear scale and side scatter (SS) on the horizontal axis in a log scale. Side scatter represents cell

granularity whereas forward scatter represents cell size. The gated area represents the red cell population, which is selected for analysis.

2nd Histogram; A single channel histogram of RNA containing red cells or reticulocytes, stained by Thiazole orange (TO) is shown. Green fluorescent signal detected by FL1 is shown on X axis in a log scale. Number of cells (cell count) is shown on Y axis in a linear scale. The cut-off point (B) was set at 0.3% above the negative population.

3rd Histogram; A single channel histogram of AM expressing red cells or AM^+cells, stained by MoAb-AM coupled with anti-mouse-globulin-RPE conjugate is shown. Red fluorescent signal detected by FL2 is shown on X axis in a log scale. Number of cells (cell count) is shown on Y axis in a linear scale. The cut-off point (C) was set at 0.3% above the negative population.

4th Histogram; A single channel histogram of HbF containing red cells or F^+cells, stained by MoAb-HbF-TC is shown. Deep-red fluorescent signal detected by FL4 is shown on X axis in a log scale. Number of cells (cell count) is shown on Y axis in a linear scale. The cut-off point (D) was set at 0.3% above the negative population.

Bottom, left→right

5th Histogram; A double channel histogram of reticulocytes and AM^+cells is shown. Green fluorescent signal (TO) detected by FL1 is shown on X axis in a log scale. Red fluorescent signal (RPE) detected by FL2 is shown on Y axis also in a log scale. The horizontal cut-off line (E) was set at 0.3% above the negative population, to separate the positive and negative AM (RPE) expressing cells. The vertical cut-off line (E) was also set at 0.3% above the negative population, to separate the positive and negative RNA (TO) or reticulocytes and mature red cells. These two lines divide the histogram into 4 quadrants (Q1-Q4). The cell number is presented in dots and dot density. **Q1** (top-left) represents AM^+mature red cells since they are positive only for AM but negative for RNA. **Q2** (top-right) represents AM^+reticulocytes since they are positive for both RNA and AM. **Q3** (bottom-left) represents ordinary mature red cells since they are negative for both

RNA and AM. **Q4** represents ordinary reticulocytes since they are positive only for RNA but negative for AM.

6th Histogram; A double channel histogram of reticulocytes and F^+cells is shown. Green fluorescent signal (TO) detected by FL1 is shown on X axis in a log scale. Deep-red fluorescent signal (TC) detected by FL4 is shown on Y axis also in a log scale. The horizontal cut-off line (F) was set at 0.3% above the negative population, to separate the positive and negative HbF (TC) or F^+cells and F^-cells. The vertical cut-off line (F) was also set at 0.3% above the negative population, to separate the positive and negative RNA (TO) or reticulocytes and mature red cells. These two lines divide the histogram into 4 quadrants (Q1-Q4). The cell number is presented in dots and dot density. **Q1** (top-left) represent F^+mature red cells since they are positive only for HbF but negative for RNA. **Q2** (top-right) represents F^+reticulocytes since they are positive for both HbF and RNA. **Q3** (bottom-left) represents ordinary mature red cells since they are negative for both HbF and RNA. **Q4** represents ordinary reticulocytes since they are positive only for RNA but negative for HbF.

7th Histogram; A double channel histogram of F^+cells and adhesion molecule expressing cells or AM^+cells is shown. Deep-red fluorescent signal (TC) detected by FL4 is shown on X axis in a log scale. Red fluorescent signal (RPE) detected by FL2 is shown on Y axis also in a log scale. The horizontal cut-off line (G) was set at 0.3% above the negative population, to separate the positive and negative AM (RPE) expressing cells. The vertical cut-off line (G) was also set at 0.3% above the negative population, to separate the positive and negative HbF (FITC) or F^+cells and F^-cells. These two lines divide the histogram into 4 quadrants (Q1-Q4). The cell number is presented in dots and dot density. **Q1** (top-left) represents ordinary AM^+cells since they are positive only for AM but negative for HbF. **Q2** (top-right) represents AM^+F^+cells since they are positive for both HbF and AM. **Q3** (bottom-left) represents ordinary red cells since they are negative for both HbF and AM. **Q4** represents ordinary F^+cells since they are positive only for HbF but negative for AM.

8th Histogram; A triple channel histogram for the combination of TO (logFL1, green, for RNA), RPE (logFL2, red, for AMs) and TC (logFL4, deep-red, for HbF) is shown. The combination of these 3 colour signals was put together into this triple colour histogram. The histogram shows cell numbers in all combinations. Gate **H** represents all 3 negative (---) population for TO, RPE and TC respectively, while gates **I** (+--), **J** (--+), **K** (+-+), **L** (-+-), **M** (++-), **N** (-++) and **O** (+++) represent the other combinations respectively. The cut-off point to separate the positive and negative populations of each channel was set at 0.3% above the negative control population, which is greater than mean plus 3 SD of negative population using samples which were either unstained or stained with irrelevant isotype control antibodies.

3.4.4. Comparison of adhesion molecule expressing cells obtained from double- and triple-colour staining

Study design

In the single-colour staining method for adhesion molecule expression on red cells and the double-colour staining method with TO for RNA in reticulocytes, fresh unfixed/unpermeabilised red cells were used. However, in the triple staining method with MoAb-HbF-TC for F^+cells, it was necessary to use fixed/permeabilised red cells to enable the MoAb-HbF-TC to pass through the red cell membrane. It is important that the fixation and permeabilisation do not disturb the adhesion molecule expression. In this section therefore, the %AM expressing cells obtained from single-colour staining is compared with the values obtained from either double-colour or triple-colour staining. In order to determine whether the additional of another 1 or 2 colour affects the AM assay.

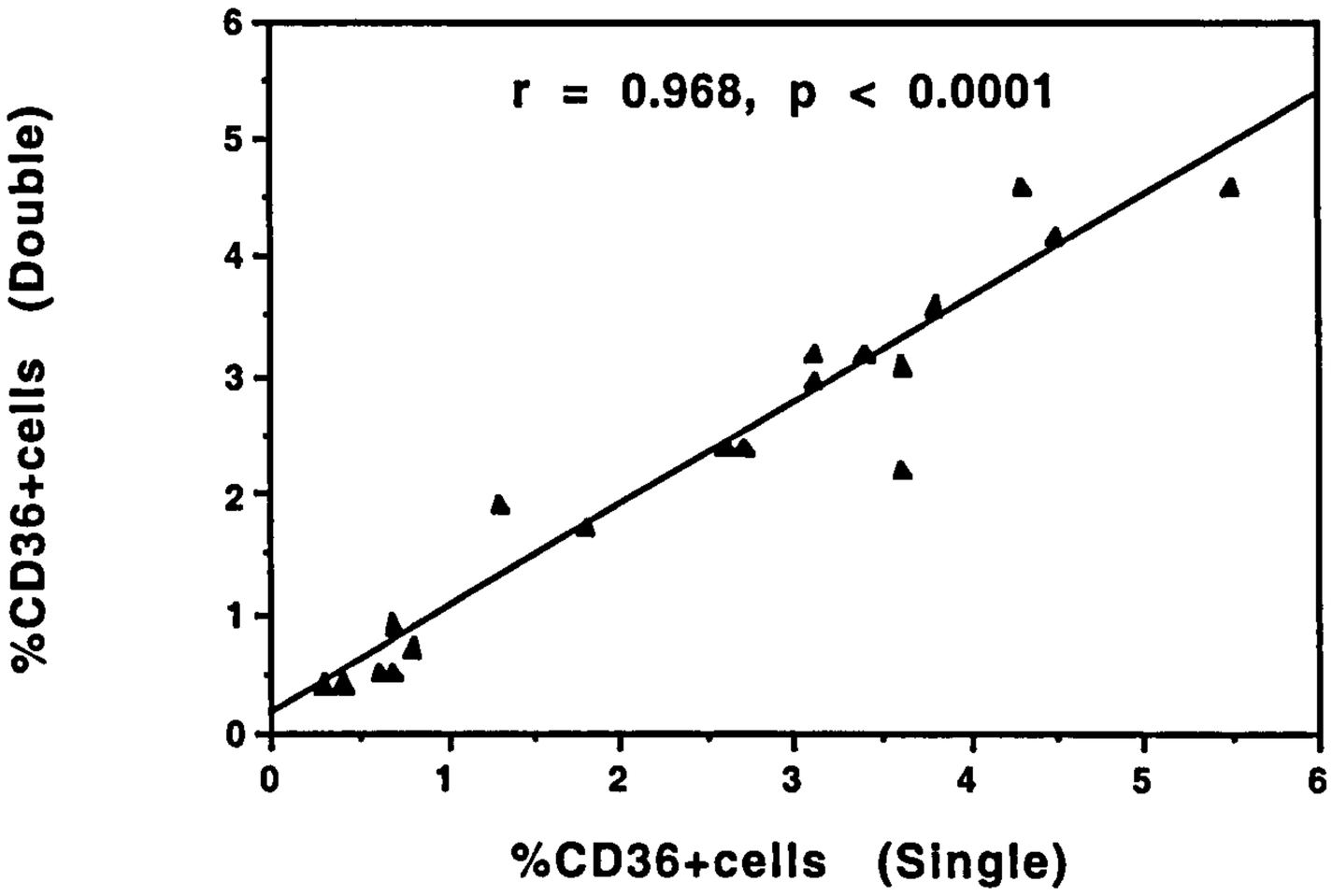
A total of 20 blood samples from 7 normal control subjects and 13 sickle cell patients were assayed in parallel for percentages of adhesion molecule (CD36, CD41 and CD49d) expressing cells on fresh (single-colour staining, section 3.2.11) and fixed/permeabilised red cells (double- and triple-colour staining, see sections 3.4.2 and 3.4.3). The overall % red cells expressing AMs

obtained from these 2 treatments (fresh and fixed/permeabilised) were compared. The correlation of coefficient (r) between these 3 methods [1 fresh (single-colour staining) and 2 fixed/permeabilised (Double- and triple-colour staining)] were also determined.

Results and discussion

The overall %$CD36^+$cells obtained from single-colour staining (fresh red cells), double-colour staining and triple-colour staining (fixed/permeabilised red cells) were 2.37±0.36, 2.20±0.33 and 2.20±0.33% respectively. The %$CD41^+$cells obtained from these methods were 2.16±0.29, 2.00±0.28 and 2.00±2.28% respectively and the %$CD49d^+$cells were 2.03±0.30, 1.93±0.30 and 1.93±0.31% respectively. There was no significant difference between %AM^+cells within the same AM. The correlation coefficient (r) of %$CD36^+$cells between single and double staining was 0.968 and between single and triple staining was 0.969 (Figure 3.4.4.1). These correlation coefficients of %$CD41^+$cells were 0.953 and 0.954 respectively (Figure 3.4.4.2) and of $CD49d^+$cells were 0.967 and 0.949 respectively (Figure 3.4.4.3). Every correlation was highly significant ($p<0.0001$).

These findings indicate that the fixing and permeabilisation has had a minimal effect on the expression of these three adhesion molecules. Therefore double- or triple-colour staining on fixed/permeabilised red cells is applicable.



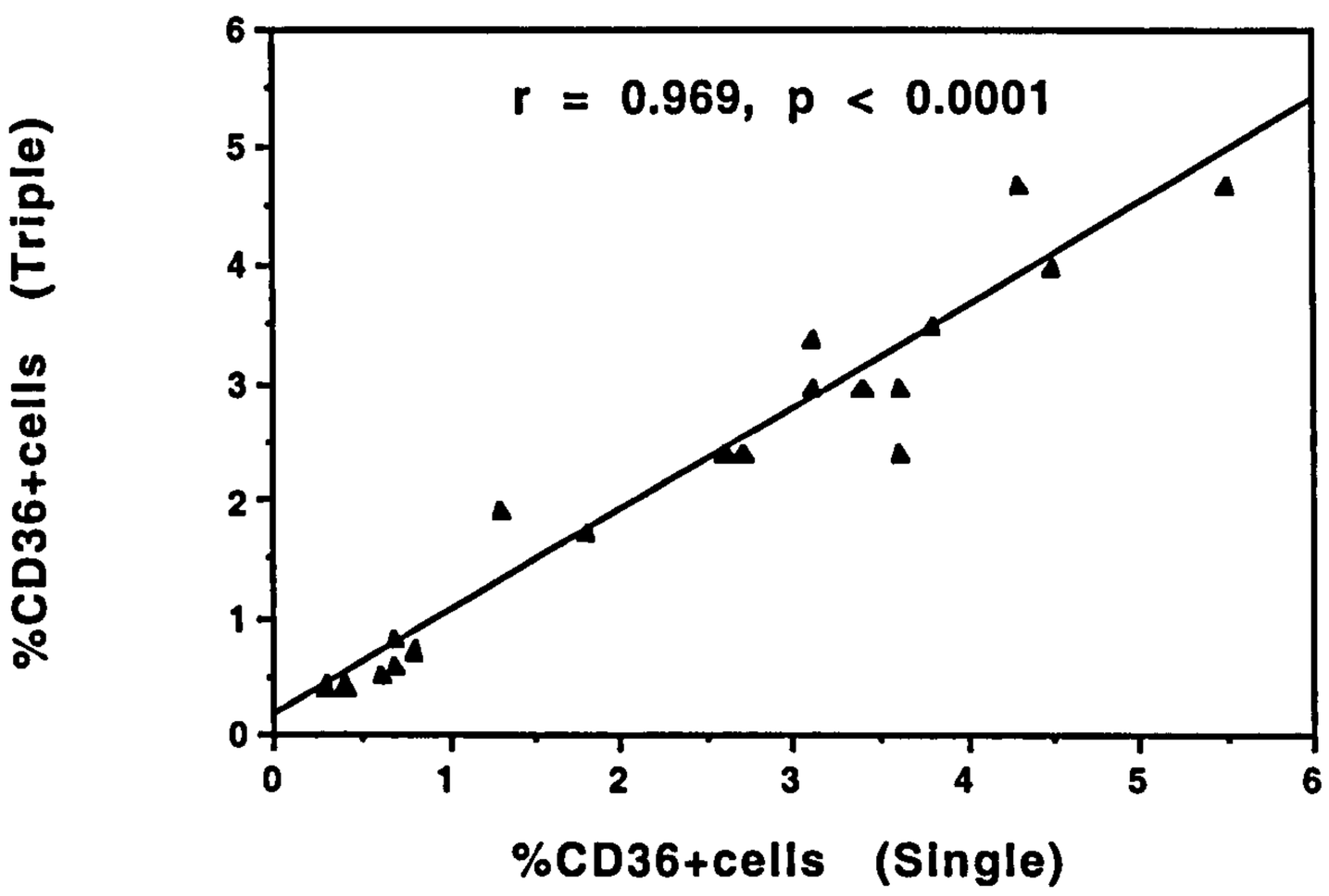
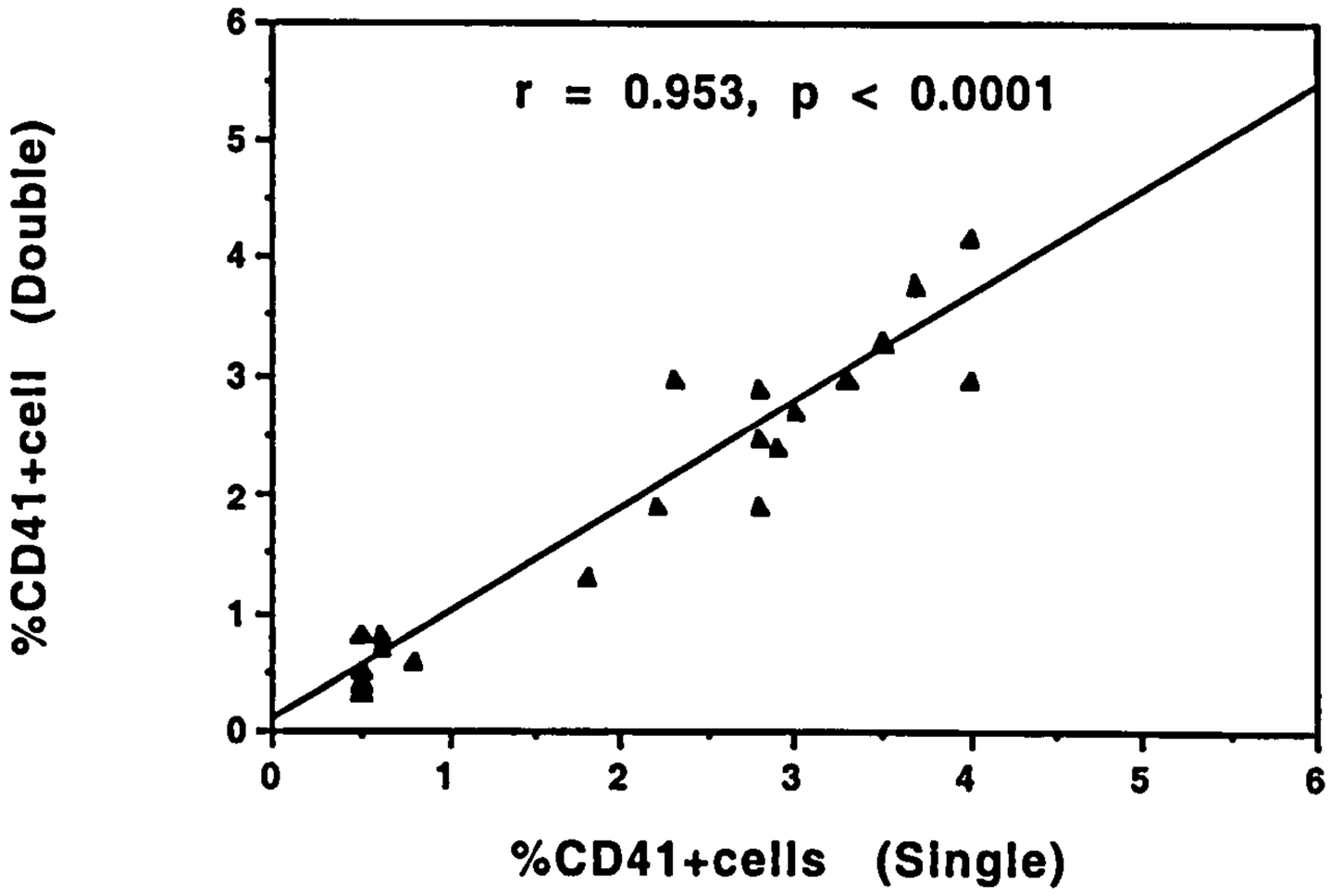


Figure 3.4.4.1 Comparison of %CD36 expressing cells obtained from fresh/unfixed and fixed/permeabilised red cells

A total of 20 blood samples were assayed for the percentages of $CD36^+$cells on fresh/unfixed (single-colour staining) and fixed/permeabilised (double- and triple-colour staining) red cells. These were collected from 7 normal donors and 13 sickle cell patients to include various levels of $\%AM^+$cells. The overall means of $\%CD36^+$cells comparing between fresh/unfixed and fixed/permeabilised red cells were not significantly different.



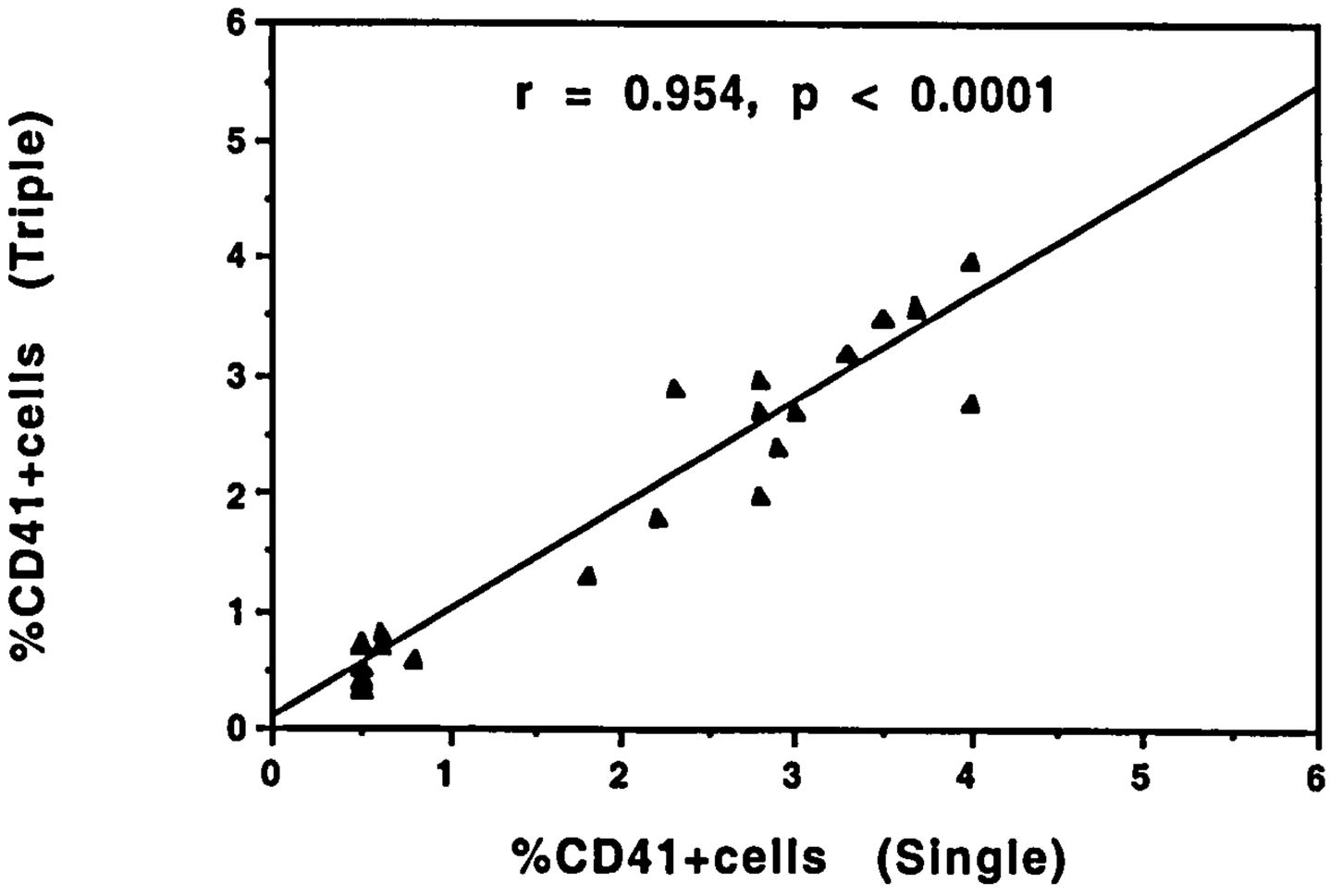
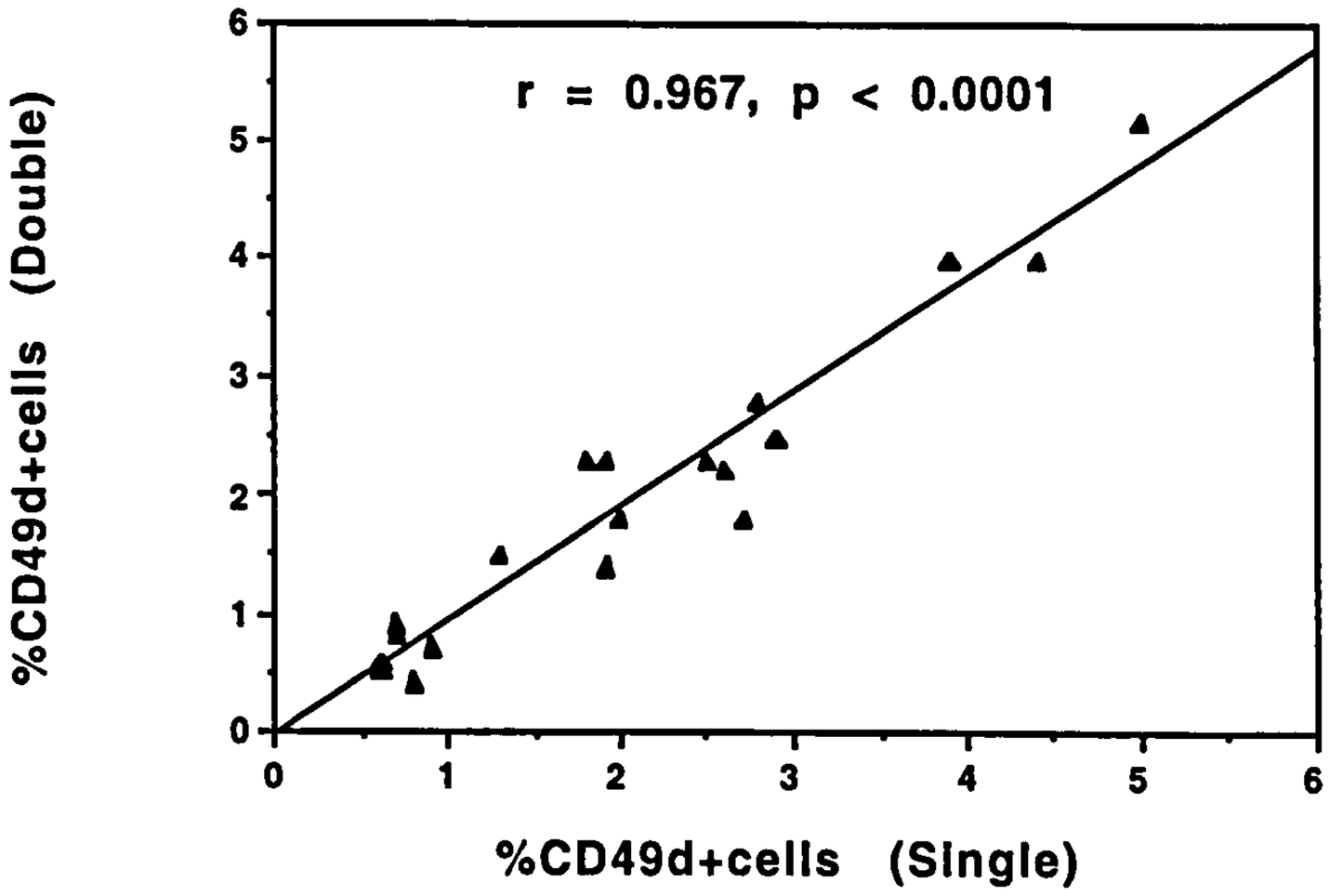


Figure 3.4.4.2 Comparison of %CD41 expressing cells obtained from fresh/unfixed and fixed/permeabilised red cells

A total of 20 blood samples, collected from 7 normal donors and 13 sickle cell patients, were assayed for percentages of $CD41^+$cells on fresh/unfixed (single-colour staining) and fixed/permeabilised (double- and triple-colour staining) red cells. The overall means of $\%CD41^+$cells comparing between fresh/unfixed and fixed/permeabilised red cells were not significantly different.



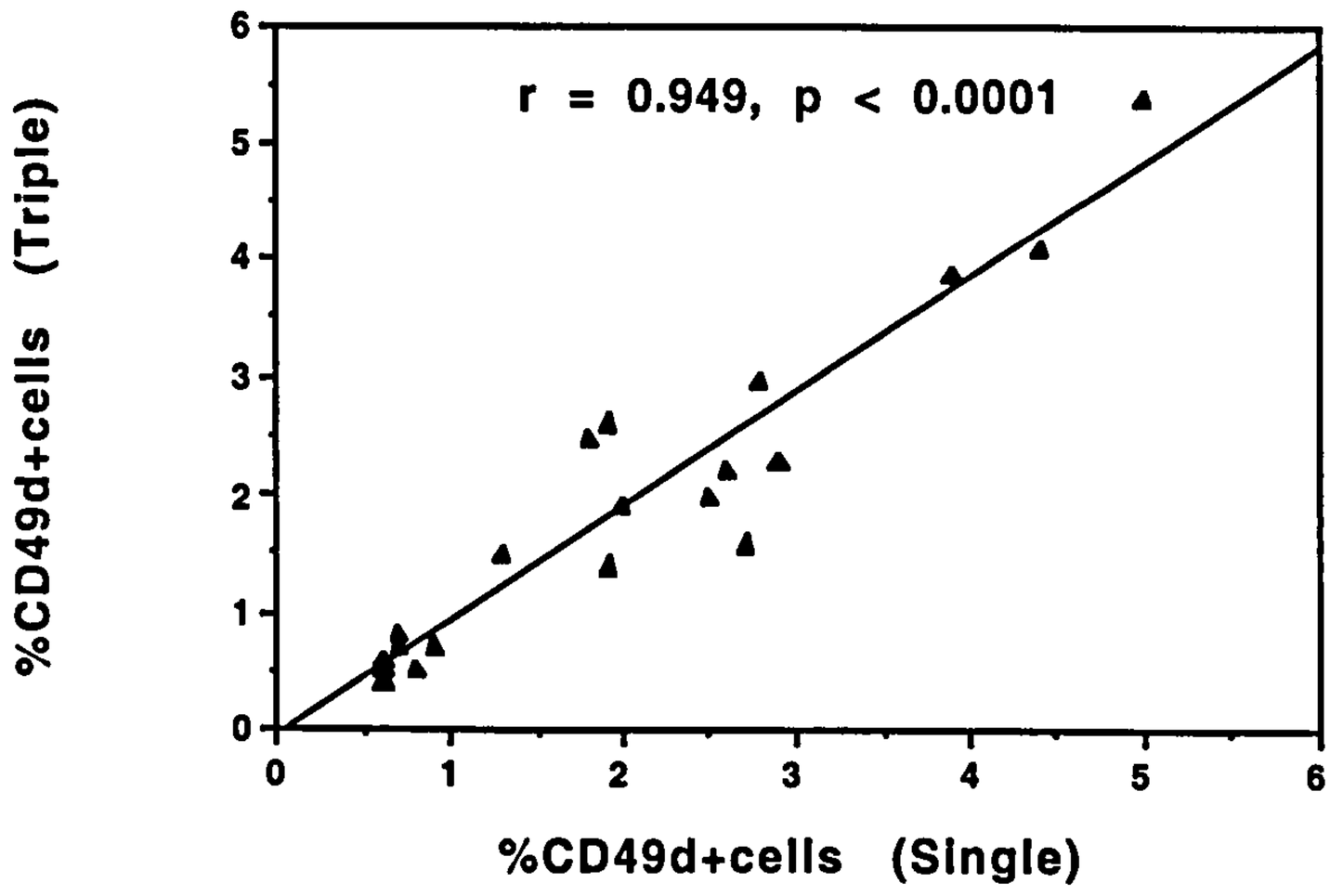


Figure 3.4.4.3 Comparison of %CD49d expressing cells obtained from fresh/unfixed and fixed/permeabilised red cells

A total of 20 blood samples, collected from 7 normal donors and 13 sickle cell patients were assayed for percentages of $CD49d^+$cells on fresh/unfixed (single-colour staining) and fixed/permeabilised (double- and triple-colour staining) red cells. The overall means $\%CD49d^+$cells comparing between fresh/unfixed and fixed/permeabilised red cells were not significantly different.

3.4.5. Effect of anticoagulants and sample stability on the adhesion molecule expressing cell assay by triple-colour staining flow cytometry

Study design

As with the single- and double-colour staining methods, it was important to find whether different anticoagulants could affect the stability of the sample and how long the sample could reasonably be stored before analysis. Three conventional and 1 special anticoagulants were evaluated. Blood was collected and stored at 4°C for 6 days in the respective anticoagulants and these samples were assayed for %AM^+cells every day for 6 days.

A blood sample from a sickle cell patient was collected in EDTA, citrate, heparin and CTAD. The samples were assayed for %AM^+cells using triple-colour staining flow cytometry (section 3.4.3) on the day of collection (day 1) and for a further 5 consecutive days (day 2–6). Samples were stored at 4°C during the study. The mean results on each day were compared.

Results and discussion

Percentages of AM^+cells in the same sample collected into different anticoagulants are not significantly different within the first 3 days of collection (Figure 3.4.5).

These results show that any of these 4 anticoagulants are suitable for the assay of AM expressing cells. Samples can be stored at 4°C for up to 3 days without any significant difference in the results.

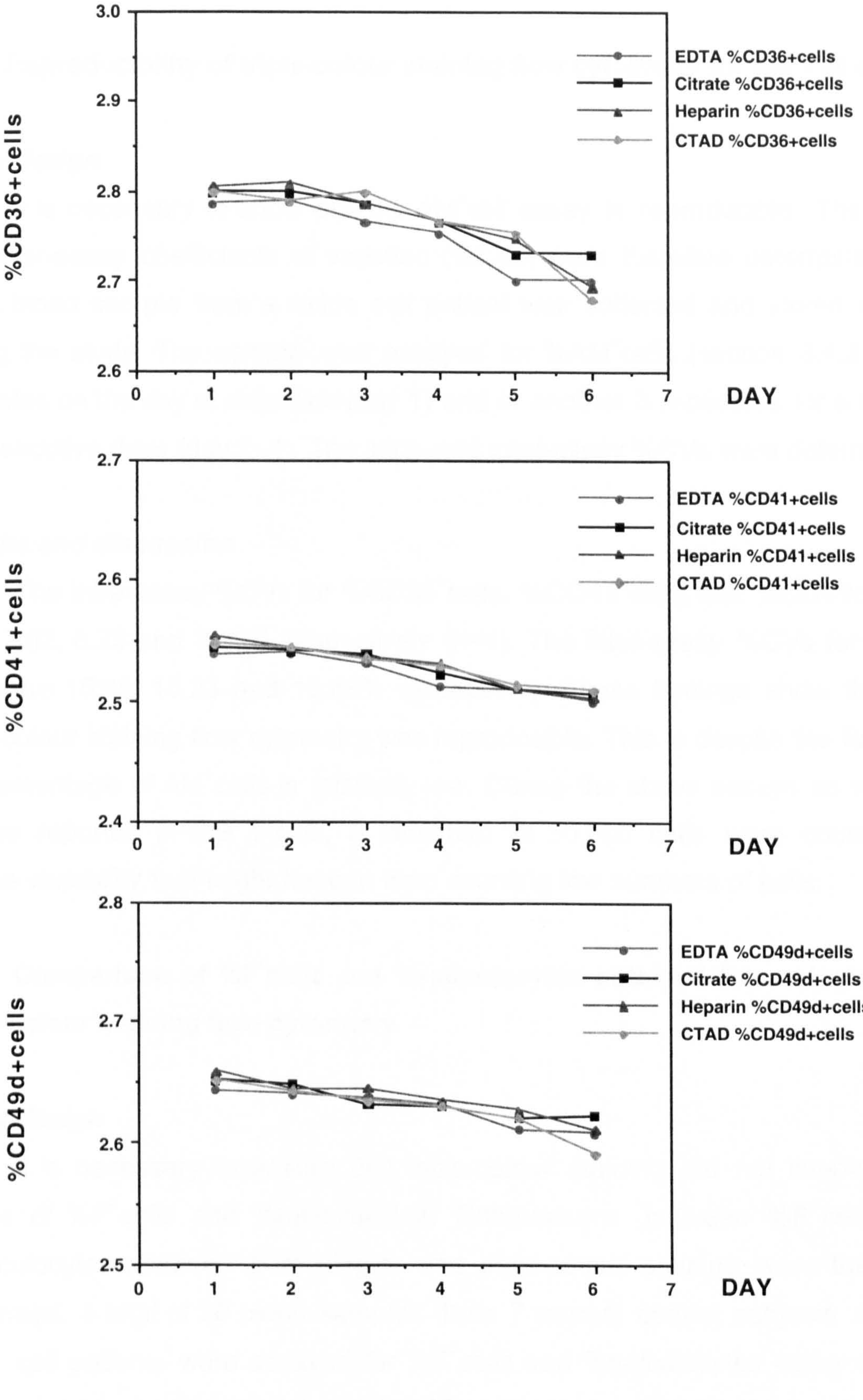


Figure 3.4.5 Percentages of AM expressing cells obtained from triple-colour staining by days of storage in different anticoagulants

See text for details.

3.4.6. Reproducibility of triple-colour staining flow cytometry for AM^+cell assay

Study design

It is necessary to show that the AM^+cell assay is reproducible. The intra- and inter-assay coefficients of variation (%CVs) were therefore determined. An EDTA blood sample from a sickle cell patient was collected and stored at 4°C during the study. The sample was assayed for $\%AM^+$cells (section 3.4.3.) in 4 replicates on the day of collection (day 1) and in another 3 replicates for a further 3 consecutive days (day 2–4). The intra- and inter-assay %CVs were determined.

Results and discussion

The intra-assay %CVs for $\%CD36^+$cells, $\%CD41^+$cells and $\%CD49d^+$cells were 7.62, 8.23 and 7.89% respectively (n=4). The inter-assay %CVs for these AMs were 15.45, 16.23 and 15.68% respectively. These findings show that the triple-colour staining flow cytometry was reproducible. This is despite the fact that the percentage of AM^+cells is relatively low. During the above assays as with all assays reported in this thesis, a minimum of 50,000 cells were counted to reduce variability that might happen from counting low numbers of cells.

3.4.7. Comparison of $\%F^+$cells and %reticulocytes obtained from single- and triple-colour staining flow cytometry

Study design

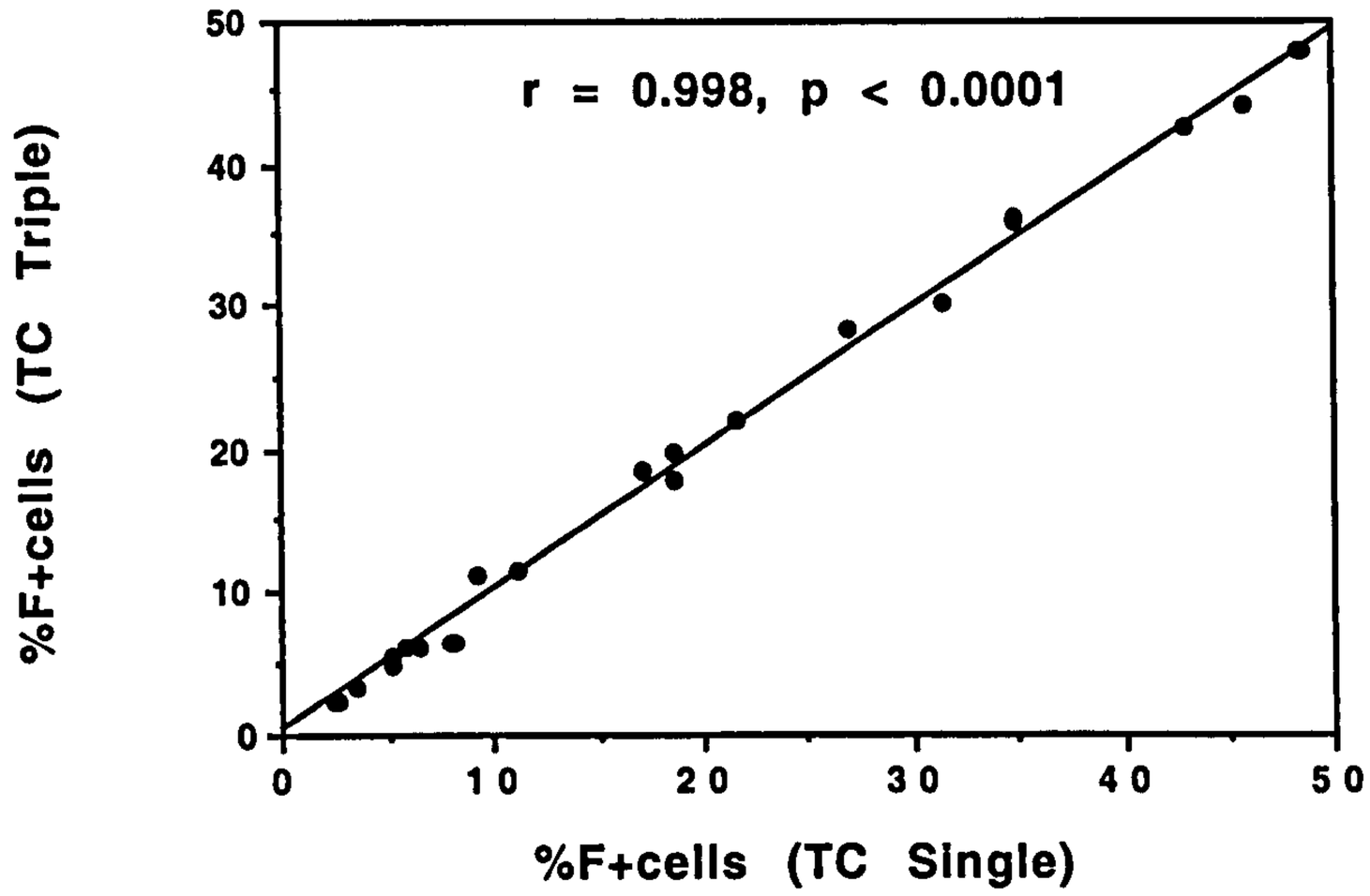
It is necessary to ensure that triple-colour staining did not interfere the values of $\%F^+$cells and %reticulocytes. Comparisons between $\%F^+$cells and %reticulocytes obtained from single- and triple-colour staining were therefore performed. A total of 20 blood samples from 7 normal control subjects and 13 sickle cell patients were assayed for $\%F^+$cells and %reticulocytes using single-colour staining (section 3.2.3 and 3.2.10 respectively) and triple staining (see sections 3.4.3). The overall $\%F^+$cells and %reticulocytes were compared. The

correlation coefficients (r) between these two methods were also determined for both %F^+cells and %reticulocytes.

Results and discussion

The overall means %F^+cells and %reticulocytes were not significantly different. The correlation coefficient (r) between these two methods for %F^+cells and %reticulocytes were 0.998 and 0.996 respectively with highly significant correlation ($p<0.0001$). (Figure 3.4.7)

These results show that the %reticulocytes obtained from the single-colour and triple-colour staining are comparable. Furthermore, the % F^+cell obtained from these two methods are also comparable. Thus, the use of the three colour together does not interfere the results of %F^+cells or %reticulocytes. Triple-colour staining are therefore valid and comparable to single-colour staining method.



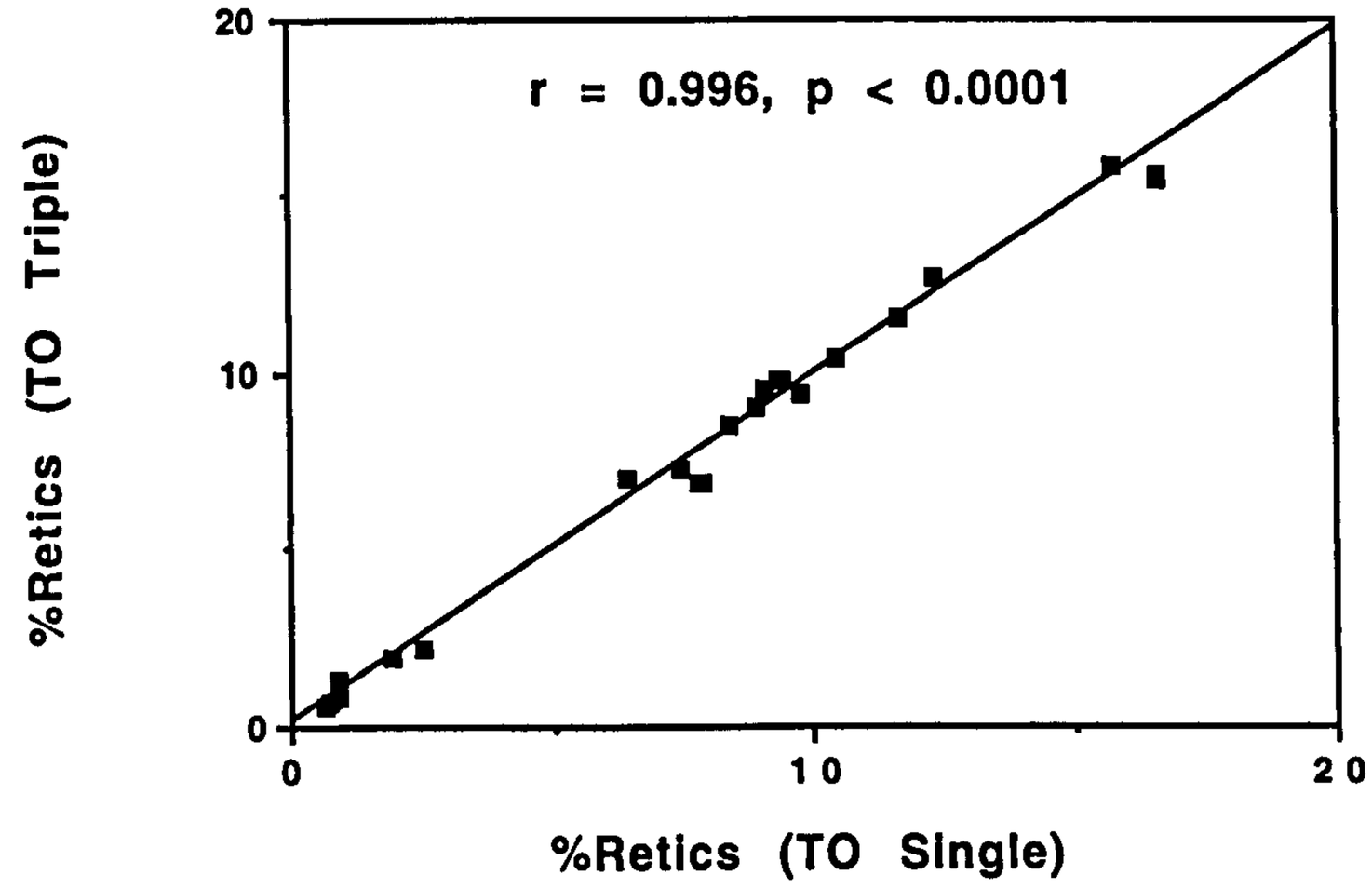


Figure 3.4.7 Correlation between %F⁺cells and %reticulocytes obtained from single-colour and triple-staining methods

A total of 20 blood samples from 7 normal control subjects and 13 sickle cell patients were assayed for %F⁺cells and %reticulocytes using single-colour staining and triple-colour staining. The overall means %F⁺cells and %reticulocytes were non significantly different.

3.5. CONCLUSIONS

In this chapter, the development of single-, double- and triple-colour staining of F^+cells, F^+reticulocytes, AM expressing cells and their co-distributions has been described. The stability of the samples in a variety of anticoagulants was demonstrated and the reproducibility of the different assays was identified. The flow cytometric methods developed in this thesis are simple, rapid, and reproducible and do not need sophisticated equipment except a flow cytometer. The methods for fixation and permeabilisation are also simple and rapid. The 3 conventional (EDTA, citrate and heparin) and one special (CTAD) anticoagulants are all suitable for these methods. Blood sample storage at 4°C is valid up to 15 days for the F^+cell assay, 6 days for the F^+reticulocyte assay and 3 days for the AM^+cell assay respectively. The simplified procedure without any washing step is even more simple and rapid and gives comparable results to the original one.

It is important to show that assay of these variables simultaneously does not interfere with the values obtained for that particular variable using single- or double-colour analysis. The results show that the reproducibility and stability obtained from single-, double- and triple-colour staining is acceptable. Furthermore, the results obtained from single-, double- and triple-colour staining are comparable showing that the triple-colour staining gives reliable data. In particular, the assay of AMs does not interfere with the simultaneous assay of F^+reticulocytes. Conversely, the value of AMs obtained is similar when these are assayed alone or in combination with F^+cells or reticulocytes. This is important for the work described in the next chapter because it is important to ensure that the results of co-distributions are valid.

In order to achieve these valid results, it is necessary to be careful of the two aspects of the analysis. Firstly the handling, permeabilisation and staining of the samples and secondly the setting up of the flow cytometer and choice of fluorochromes for single-, double- and triple-colour staining. Therefore the optimal gating and colour compensations to prevent interference from one colour into another were important. This approach has been described in chapter 2.

CHAPTER 4

CONTRIBUTION OF FOETAL HAEMOGLOBIN AND RED CELL ADHESION MOLECULES TO SICKLE RED CELL SURVIVAL

4.1. F^+CELLS, F^+RETICULOCYTES AND ENRICHMENT RATIO

4.1.1. Background

The method for quantitation of co-distribution of foetal haemoglobin (HbF) and reticulocytes has been described in Chapter 3. It is now possible to address in principle whether HbF containing red cells (F^+cells) survive beyond the reticulocyte stage better than non-F cells or F-negative cells (F^-cells). F^+cells and HbF containing reticulocytes (F^+reticulocytes) are increased in the majority of sickle cell patients. Previous methodology using microscopic single-cell radial immunodiffusion has shown that the ratio of the proportion of F^+mature red cells (F^+MRCs) to the proportion of F^+reticulocytes, so called enrichment ratio (ER), is close to unity (1.00) in normal healthy subjects (Dover *et al.*, 1978a). This suggests that F^+cells do not have any survival advantage in normal individuals. In sickle cell patients, there is generally a higher proportion of F^+MRCs than F^+reticulocytes, with the ER varying from 0.7 to 5.0 (Dover *et al.*, 1978b). This is consistent with the hypothesis that F^+cells in sickle cell patients have a survival advantage over non-F cells (F^-cells) due to the slower rate of HbS polymerisation when intra-erythrocyte HbF are present (Bertles & Milner, 1968).

Another question is whether in patients receiving hydroxyurea (HU) treatment, this enrichment ratio differs significantly. Hydroxyurea is known to increase HbF and F^+cells in a substantial proportion of sickle cell patients but may also act through additional or alternative mechanisms such as decreasing numbers of white cells, reticulocytes and red cells expressing adhesion molecules. The double-colour staining flow cytometry for quantitation of F^+cells, reticulocytes and F^+reticulocytes can theoretically show whether any survival

advantage for F^+cells beyond the reticulocyte stage is enhanced or diminished by hydroxyurea treatment.

4.1.2. Distribution of F^+cells

Rationale

In chapter 3, a simplified procedure for F^+cell assay was developed and validated. In this section, the $\%F^+$cells are compared between normal donors and sickle cell patients in steady state, in vaso-occlusive painful crisis or receiving hydroxyurea therapy. The proportion of F^+cells is also compared with the proportion of HbF assayed by HPLC. Patients who had recently received red cell transfusion, as evidenced by the presence of HbA using HPLC analysis, were excluded from this study.

Study design

Sixty two blood samples were obtained from 25 normal donors (control), 18 sickle cell patients in steady state (sickle std, no vaso-occlusive painful crisis and no hospitalisation at the time of blood sampling), 12 sickle cell patients in vaso-occlusive painful crisis (sickle cri, admitted to the hospital due to a painful crisis at the time of blood sampling) and 7 sickle cell patients undergoing hydroxyurea therapy (sickle HU, 0.5-2.0 g/day, for several weeks and having a steady level of %HbF at the time of blood sampling). None of the sickle cell patients had any HbA as measured by HPLC, confirming that they had not received any recent blood transfusion (at least within 4 months before blood sampling).

The blood samples were collected into either EDTA, citrate or heparin vacutainers and were usually assayed within 2 weeks of 4°C storage, since the results in chapter 3 show that any anticoagulant is suitable for the assay and the samples were stable for 2 weeks at 4°C. Analysis of $\%F^+$cells was carried out using the simplified procedure (section 3.2.3) and HbF was measured by HPLC (section 2.5.2).

Results and discussion

In 25 control subjects the %HbF by HPLC was 0.96±0.55% and the %F^+cells 3.03±0.36% respectively. In 18 sickle std, these values were 4.06±0.77% and 17.57±2.81% respectively. In 12 sickle cri these values were 2.92±0.51% and 14.77±1.83% respectively. In 7 sickle HU these values were 15.83±2.00% and 54.09±8.45% respectively. (Figure 4.1.2)

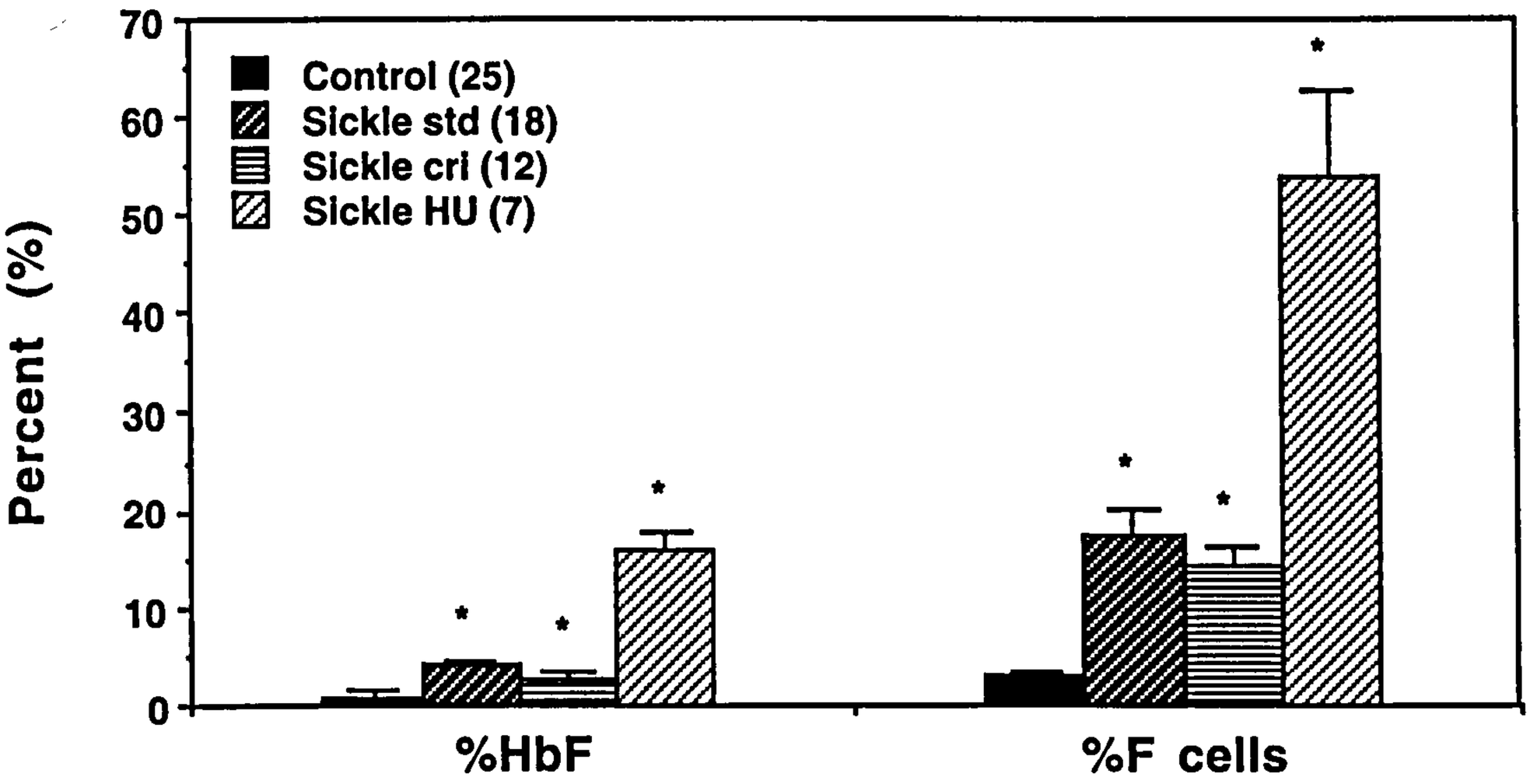


Figure 4.1.2 Percentages of HbF and F^+cells

Sixty two blood samples obtained form 25 normal donors (control), 18 sickle cell patients in steady state (sickle std), 12 sickle cell patients in crisis (sickle cri) and 7 sickle cell patients on hydroxyurea therapy (sickle HU) were assayed for %HbF using HPLC and %F^+cells using the simplified procedure. These two parameters in all three groups of patients were significantly higher than in the control group. (* significant higher than controls $p<0.01$, ** significantly higher than SS.std. $p<0.001$)

The %HbF in normal controls (0.96±0.55%) is consistent with previous reports of not more than 2% (Rochette *et al.*, 1994). Similarly the %F^+cells in normal donors of 3.03±0.36% is consistent with the levels reported by other investigators using flow cytometry, such as 3.1% (Thorpe *et al.*, 1994) and 4.4%

(Rochette *et al.*, 1994) but appeared to be lower than 7% reported using immunofluorescent staining on blood smear (Wood *et al.*, 1975).

In all 3 sickle cell patient groups, both %HbF and %F^+cells were significantly higher than in control subjects ($p<0.01$ and $p<0.001$ respectively). As expected, there was a marked increase in the %HbF and the %F^+cells in sickle patients receiving hydroxyurea therapy, consistent with previous findings using other techniques (non-flow cytometry) (Bridges *et al.*, 1996). The results confirm that F^+cell production in sickle cell patients is higher than in normal control donors and the hydroxyurea treatment in sickle cell patients can induce very high levels of both %HbF and %F^+cells.

4.1.3. Distribution of F^+cells, reticulocytes and F^+reticulocytes

Rationale

Reticulocytes are typically increased in sickle cell patients because of the compensation of their haemolytic anaemia. As shown in sections 4.1.2, F^+cells are increased in patients with increased HbF levels. Measurement of the %F^+reticulocytes (% of reticulocytes which contain HbF in the total reticulocytes) has been used previously to detect an early response to agents which increase HbF (Dover *et al.*, 1978a). In response to HU, it would be expected that increases in F^+reticulocytes would be detected before increases in HbF or F^+cells, since F^+reticulocytes represent cells recently released from the bone marrow. On the other hand, HU may decrease the %reticulocytes by decreasing haemolysis or by decreasing proliferation in the bone marrow. In this section, the percentages of F^+cells, reticulocytes, F^+reticulocytes and HbF are compared between control subjects and sickle cell patients (both on and off HU therapy) to determine whether the measurement of F^+reticulocytes by flow cytometry adds any useful information over the measurement of F^+cells and reticulocytes separately.

Study design

Sixty seven blood samples were obtained from 20 normal donors (control), 23 sickle cell patients in steady state (sickle std), 18 sickle cell patients in crisis (sickle cri) and 6 sickle cell patients receiving hydroxyurea therapy (sickle HU, 0.5 -2.0 g/day for several weeks and having a steady level of %HbF at the time of blood sampling). All sickle cell patients showed an absence of HbA by HPLC confirming that none had received recent blood transfusion. The blood samples were collected into either EDTA, citrate or heparin vacutainers, stored at 4°C and usually assayed within 5 days after collection. These anticoagulants are all suitable for the method and such samples are stable up to 5 days at 4°C (section 3.3.3). The samples were assayed for HbF levels using HPLC (section 2.5.2) and simultaneous measurements of $\%F^+$cells, %reticulocytes and $\%F^+$reticulocytes (section 3.3.1). The %HbF (% of HbF in total Hb in the hemolysate), $\%F^+$cells (% of F^+cells in total red cells), %reticulocytes (% of reticulocytes in total red cells) and $\%F^+$reticulocytes (% of F^+reticulocytes in total reticulocytes) of these four groups were determined and compared.

Results and discussion

In the 20 controls, %HbF was 1.25±0.13%, $\%F^+$cells was 3.25±0.45%, %reticulocytes was 1.39±0.10% and $\%F^+$reticulocytes was 3.31±0.41% respectively. In 23 sickle std, these values were 6.07±0.86%, 28.55±3.37%, 12.19±1.40% and 13.19±1.54% respectively. In 18 sickle cri, these values were 5.05±1.16%, 20.89±3.33%, 11.61±1.40% and 18.70±2.20% respectively. In 6 sickle HU, these values were 21.68±3.40%, 63.97±5.5%, 4.94±0.81% and 34.08±3.71% respectively. (Figure 4.1.3)

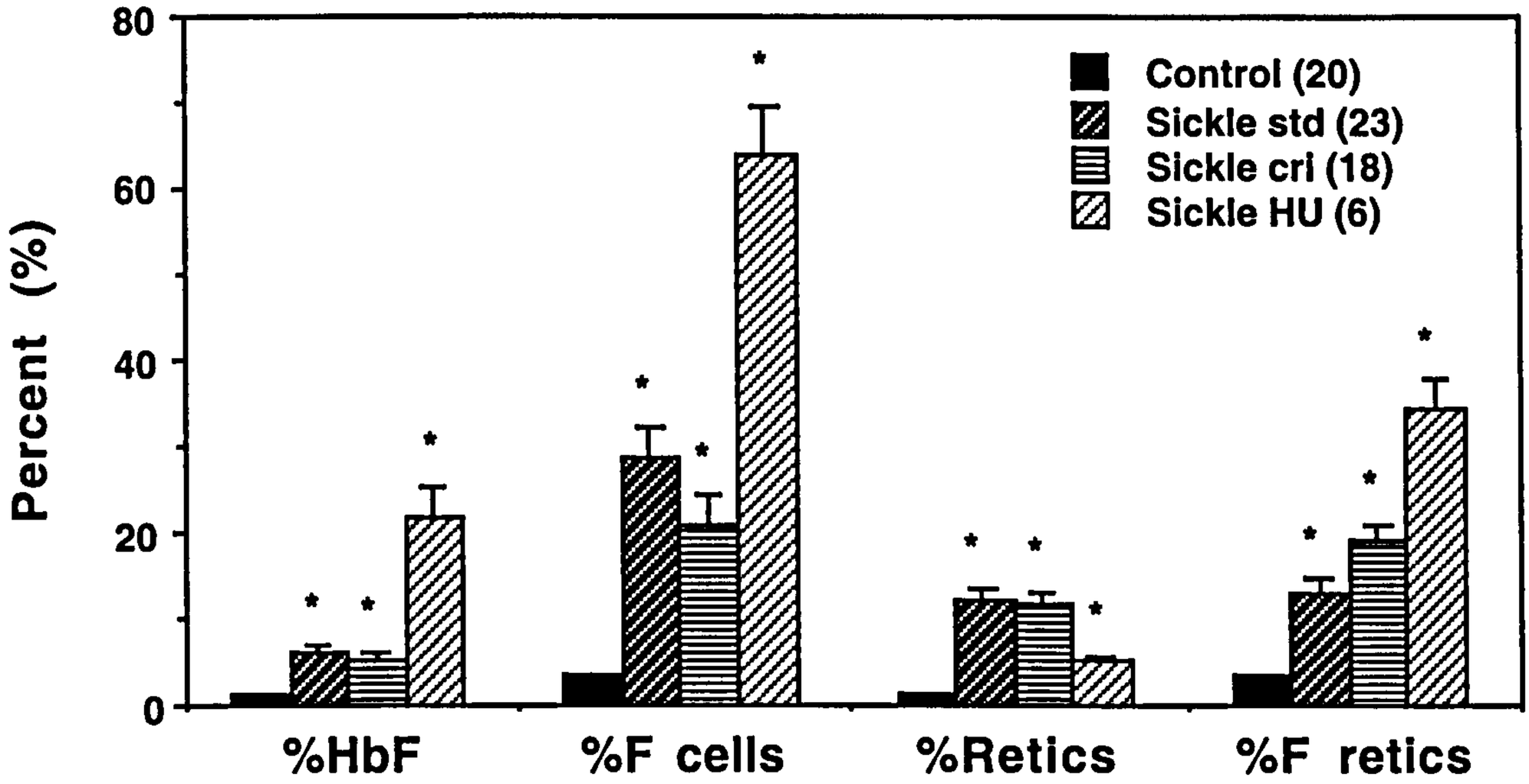


Figure 4.1.3 Percentages of HbF, F⁺cells, reticulocytes and F⁺reticulocytes

Sixty seven blood samples obtained from 20 normal donors (control), 23 sickle cell patients in steady state (sickle std), 18 sickle cell patients in crisis (sickle cri) and 6 sickle cell patients on hydroxyurea therapy (sickle HU) were assayed for %HbF by HPLC and %F⁺cells, %reticulocytes and %F⁺reticulocytes by double-colour staining flow cytometry. These four parameters in all three groups of patients were significantly higher than in the control group (*$p<0.01$). The %HbF, %F⁺cells and %F⁺reticulocytes in HU-treated patients were significantly higher than in sickle std at $p<0.001$. The %reticulocytes in HU-treated patients was significantly lower than in sickle std at $p<0.05$. Retics or retics = reticulocytes

There are significantly higher levels of all four parameters (%HbF, %F⁺cells, %reticulocytes and %F⁺reticulocytes) in sickle cell patients over normal control subjects ($p<0.01$). However, there is no significant difference in these values between sickle cell patients in steady state and in crisis. In HU-treated sickle cell patients, %HbF, %F⁺cells and %F⁺reticulocytes were very high, consistent with a previous report, which used microscopic single-cell radial immunodiffusion for F⁺reticulocyte measurement (Charache *et al.*, 1992). It is noteworthy that %reticulocytes are significantly decreased in the HU-treated patients compared to non-HU-treated patients ($p<0.05$). This decrease has been

observed by other investigators (Dover *et al.*, 1986; Ferster *et al.*, 1996) and is due to the cytostatic effect of HU.

In patients who have been on HU for several weeks, the increase in $\%F^+$cells is more marked than that of $\%F^+$reticulocytes, possibly because F^+cells survive longer than F^-cells and supression of bone marrow by HU leads to reduce production of reticulocytes and also F^+reticulocytes. Therefore in HU-treated patients, there is a lower proportion of young red cells containing HbF than mature red cells containing HbF. Since these patients had been on HU for several weeks, the observations represent not the initial response to HU, where F^+reticulocytes might be increased before a general increase in F^+cells was detected, but this is a situation where the majority of F^+reticulocytes have already matured into F^+mature red cells. Therefore, the fact that the % of F^+mature red cells exceeds the % of F^+reticulocytes suggests that F^+cells have a survival advantage over cells which lack HbF (F^-cells) in sickle cell patients. In contrast, the $\%F^+$cells is approximately the same as the $\%F^+$reticulocytes in control subjects, consistent with they're being no survival advantage of F^+cells in control subjects. Thus the ER (ratio of $\%F^+$mature red cells to $\%F^+$reticulocytes) gives an indication of the survival advantage of cells containing HbF. This ratio was first reported by Dover *et al.* (1978a) using microscopic single-cell radial immunodiffusion techniques but has not been reported for HU treated patients. In the subsequent sections, this ratio is examined systematically.

4.1.4. F^+cell enrichment ratio (ER)

Study design

An advantage of the double-colour staining for simultaneous measurements of $\%F^+$cells, %reticulocytes and $\%F^+$reticulocytes over single-colour staining for F^+cell measurement alone is the ability to determine the ER. Dover *et al.* (1978a) reported an increased ER in sickle cell patients ranging from 0.7-5.0. In order to confirm this finding using flow cytometric method, the ER was

determined in normal control subjects and in sickle cell patients. The F^+cell ER was calculated from the data obtained in section 4.1.3 using the formula below.

$$\text{Enrichment ratio} = \frac{\text{Proportion (\%) of } F^+\text{mature red cells in total mature red cells}}{\text{Proportion (\%) of } F^+\text{reticulocytes in total reticulocytes}}$$

Results

The ER in the normal control subjects (n=20) was 0.999±0.038. In non-transfused sickle cell patients in steady state (n=23) was 2.186±0.145. In sickle cell patients in crisis (n=18) was 1.780±0.189. In sickle cell patients undergoing hydroxyurea therapy (n=6) was 1.610±0.259 respectively. (Figure 4.1.4)

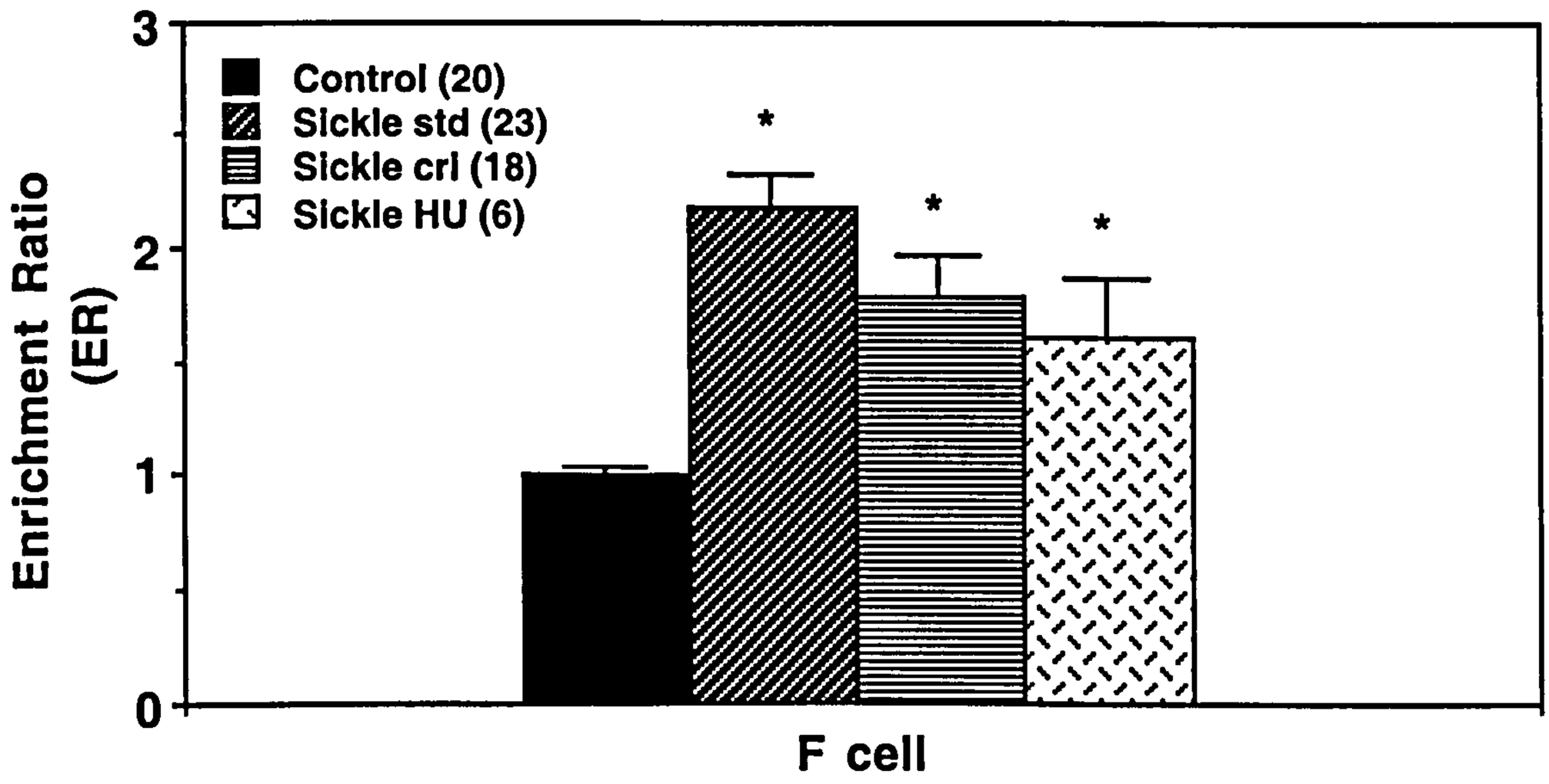


Figure 4.1.4 The enrichment ratio (ER) of F^+cells

The ERs of 20 normal donors (control), 23 sickle cell patients in steady state (sickle std), 18 sickle cell patients in crisis (sickle cri) and 6 sickle cell patients on hydroxyurea therapy (sickle HU) were calculated from the data in section 4.1.3. The ER in all three groups of patients was significantly higher than in the control group. (* $p<0.005$)

Discussion

The F^+cell enrichment ratio (ER) in normal control subjects was close to unity (1.000), which indicates that there is no preferential survival of the F^+cells over F^-cells (normal HbA containing cells without HbF). This result confirms the previous findings (Dover *et al.*, 1978a). In all 3 groups of sickle cell patients the ER was significantly higher than in the control subjects ($p<0.005$), which was also consistent with Dover's finding, but the method used in this thesis is more convenient. These results indicate that there is a survival advantage of F^+cells in sickle cell patients. In sickle cell patients, a higher ER indicates a higher proportion of F^+cells mature beyond the reticulocyte stage and on average they have an approximately two fold ER over normal control subjects. There is no significant difference between ER of SS.std. and SS.cri., but the ER in SS.HU is lower than in SS.std. (just reach the significant level, $p<0.05$). This finding indicates that at the peak of HbF production induced by HU, the difference between $\%F^+$cells and $\%F^+$reticulocyte is closer than in untreated patients. This is probably because in HU-treated patients, a higher proportion of red cells are F^+cells when compared to non-treated patients. Therefore, the ER becomes more closer to unity (1.000). If HU has an absolute effect on inducing all newly released reticulocytes to become F^+reticulocytes, shortly afterwards the mature red cell population would all become 100% F^+cells. So the ER would be 1.000 according to the formula. This phenomenon is probably found in homozygous HPFH individuals, who produce all 100% F^+cells.

4.2. ADHESION MOLECULE EXPRESSING RED CELLS, RETICULOCYTES AND F^+CELLS

4.2.1. Background

The relationship between AM expression and HbF content on red cell survival has not been previously reported and is potentially important for two reasons. Firstly, it may provide information about the nature of F^+cells in normal individuals, in particular whether proportion of AM expressing F^+cells is

increased or decreased and whether such cells survive normally. Secondly, in sickle cell disorders it is not known whether the co-expression of HbF and AMs accounts for the better survival advantage of F^+cells compared to F^-cells (red cells containing only HbS without HbF).

In this section, the proportion of red cells expressing AMs has been compared in total red cells, mature red cells, reticulocytes, F^+cells and F^-cells in healthy control subjects and sickle cell patients. The AMs examined were CD36, CD41 and CD49d. Three double-colour staining flow cytometric assay was employed (section 3.4.2).

4.2.2. Proportions of total red cells, reticulocytes and F^+cells expressing adhesion molecules

Rationale

The purpose of the work in this section was to examine two questions. Firstly whether AM expression is greater on reticulocytes than on mature red cells? Secondly whether AMs are expressed to the same degree on F^+cell and F^- cell populations? Previous investigators, have found that stress reticulocytes in patients with sickle cell disease express high levels of AMs, especially CD36 (Browne & Hebbel, 1996). However these investigators did not examine the distribution of other AMs (CD41 and CD49d) on reticulocytes in controls and in sickle cell patients. In this section, 3 AMs (CD36, CD41 and CD49d) were therefore examined with respect to their co-distribution on F^+cells and on reticulocytes. These co-distributions were then compared between sickle cell patients and control subjects.

Study design and methodology

Seventy blood samples were obtained from 23 normal donors (Control), 23 non-transfused sickle cell patients in steady state (Sickle std), 14 non-transfused sickle cell patients in crisis (Sickle cri) and 10 sickle cell patients undergoing hydroxyurea therapy (sickle HU, 0.5-2.0 g/day for several weeks and

reaching a steady level of %HbF at the time of blood sampling) at University College Hospitals, London, UK. The percentages of AM expressing cells in the total population of red cells, in the reticulocyte population and in the F^+cells population were determined using the three separated double-colour staining flow cytometric assays (section 3.4.2). Blood samples were obtained in either EDTA, citrate or heparin vacutainers, stored at 4°C and assayed within 3 days.

Results

The results show that in sickle cell disorders, the %AM^+cells is increased in the total red cell and in the F^+cell populations but not in the reticulocyte population, when compared to the control subjects (Figure 4.2.2). The trends are similar for CD36, CD41 and CD49d. Therefore, the following discussion refers to all 3 AMs.

AM expression in total red cells and reticulocytes

In normal controls (n=23), the percentages of total red cell expressing CD36, CD41 and CD49d were 0.65±0.03, 0.65±0.03 and 0.69±0.03% respectively. The percentages of total reticulocyte expressing these AMs were 21.58±0.87, 21.03±1.04 and 22.19±0.90% respectively.

In steady state sickle cell patients (n=23) the % of total red cell expressing these AMs were 2.89±0.21, 2.65±0.21 and 2.77±0.26% respectively, which was increased compared to the control subjects ($p<0.001$). However, the % of total reticulocyte expressing AMs (22.23±1.59, 19.48±1.51 and 19.65±1.52% respectively) was not significantly different from the control subjects. Thus the increased % of AM^+cells in the total population of red cells in steady state sickle cell patients reflects the higher proportion of reticulocytes (11.47±0.96%) compared to the controls (1.53±0.05%), does not reflect a difference in % of reticulocyte expressing AMs.

In sickle cell patients in crisis (n=14), % of total red cells expressing these AMs and % of total reticulocytes expressing the same AMs are similar to the steady state patients. The % of total red cells expressing CD36, CD41 and

CD49d were 2.36±0.22, 2.16±0.23 and 2.25±0.21% respectively and the % of total reticulocytes expressing these AMs were 22.19±3.24, 21.41±3.76 and 19.63±3.14% respectively. The % of reticulocytes in the crisis patients (9.20±0.92%) were also increased to a similar level as in steady state patients.

In HU-treated sickle cell patients (n=10), the % of total red cell expressing these AMs (1.82±0.57, 1.92±0.35 and 1.56±0.12% respectively) was decreased to about two thirds of the levels in steady state patients. The % of reticulocytes was also decreased in HU-treated patients (5.38±0.89%) compared to the steady state patients (11.47±0.96%, $p<0.05$). However, the % of reticulocytes expressing these AMs (19.91±4.66, 18.02±3.83 and 16.92±2.58% respectively) was slightly decreased with no significant difference to either the control subjects or steady state or crisis patients.

AM expression in F^+cells

In control subjects, the % of F^+cell expressing AMs (1.14±0.05, 1.13±0.04 and 1.15±0.04% for CD36, CD41 and CD49d respectively) appears to be greater than % of total red cells expressing AMs (0.65±0.03, 0.65±0.03 and 0.69±0.03% respectively) ($p<0.01$).

In steady state patients, the % of F^+cells expressing these AMs (2.46±0.26, 2.39±0.26 and 2.36±0.26% respectively) was significantly higher than control subjects ($p<0.05$), but not significantly different from the % of total red cells expressing these AMs (2.89±0.21, 2.65±0.21 and 2.77±0.26% respectively). Thus, unlike control subjects, the % of F^+cells expressing AMs is about the same as the % of total red cells expressing AMs. In vaso-occlusive crisis patients, the % of F^+cells expressing these AMs were 2.39±0.27, 2.58±0.26 and 2.01±0.17% respectively. These values are significantly higher than the values in control subjects ($p<0.01$) but not significantly different from the values in steady state patients. In HU-treated patients, the % of F^+cells expressing these AMs (1.49±0.10, 1.72±0.09 and 1.35±0.10% respectively) was significantly reduced ($p<0.05$) compared to the steady state and crisis patients and was not significantly different from the control subjects. (Figure 4.2.2).

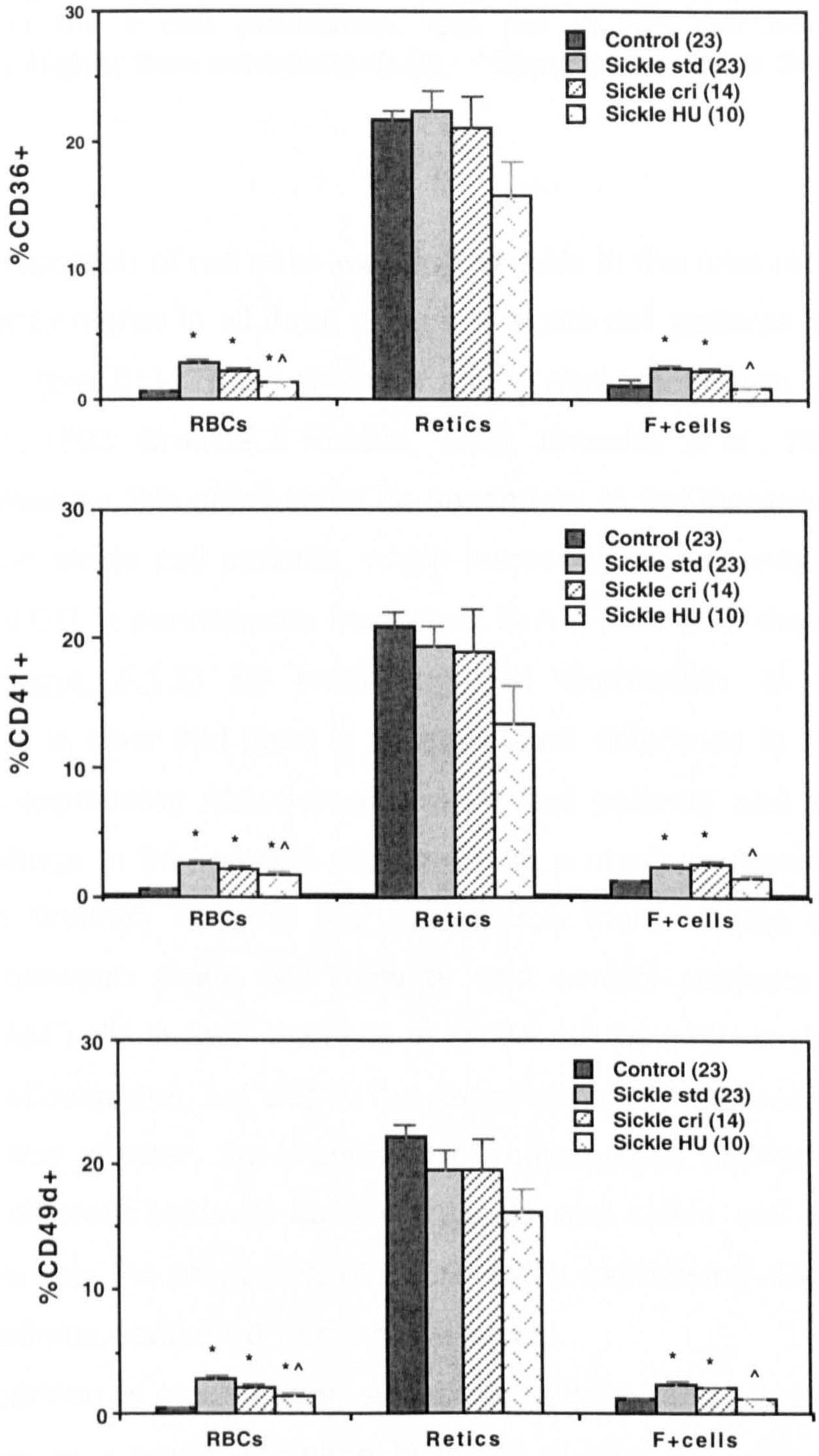


Figure 4.2.2 Percentages of cells expressing adhesion molecules in total RBCs, reticulocytes and F^+cells

A total of 70 blood samples from 23 normal donors (control), 23 sickle cell patients in steady state (sickle std), 14 sickle cell patients in crisis (sickle cri) and 10 sickle cell patients receiving hydroxyurea therapy (sickle HU) were assayed for the proportion of cells expressing CD36 (upper), CD41 (middle) and CD49d

(lower) in total red cells, reticulocytes and F^+cells using three double-colour staining flow cytometry. In sickle disorders, the % of AM^+cells is increased in total RBCs and in the F^+cell population, but not in the reticulocyte population. (*Significantly higher than controls $p<0.01$, ^Significantly lower than the sickle std $p<0.05$).

Discussion

The proportion of red cells expressing AMs in the total red cell population was significantly higher in all three groups of sickle cell patients compared to the control group ($p<0.01$). These findings are in agreement with previous reports (Brittain *et al.*, 1993; Browne & Hebbel, 1996; Joneckis *et al.*, 1993; Sugihara *et al.*, 1992). However, this effect could be secondary to the increased proportion of reticulocytes in sickle cell patients, which increased significantly over the control subjects ($p<0.01$) in parallel with increases in AM^+cells over the control subjects ($p<0.01$). (Figure 4.1.3) By examining AM expression in the reticulocyte populations, it is clear that there is no significant difference in the proportion of reticulocytes expressing AMs between sickle cell patients and control subjects. Thus the findings in this section disagree with a previous report by Styles et al. 1997. These findings suggest that qualitatively there is little difference in AM expression between sickle cell patients and control subjects. Therefore, the increase in AM^+cells in total red cells in sickle cell patients is dependent on the increase in reticulocytes, not due to the reduction of AM expressing reticulocytes. However in this chapter, the proportion of reticulocytes expressing AMs is not significantly different between control subjects and sickle cell patients. Thus, it clearly shows that the proportion of reticulocytes expressing AMs is unaltered in sickle cell patients compared to control subjects.

Comparison of steady state sickle cell patients and HU-treated sickle cell patients, there is a small decrease in the % of total red cells expressing AMs ($p<0.05$). This is consistent with the previous finding that HU treatment not only increases HbF level but also reduces sickle red cell adhesion to endothelium (Noguchi *et al.*, 1993). HU treatment also reduces soluble AMs in the plasma of sickle cell patients (Saleh *et al.*, 1998; Saleh *et al.*, 1999). Styles et al (1997) reported that the reduction of AM expressing red cells with HU treatment was not

due to the decrease in reticulocytes. However, the findings in this study suggest that the decrease in AM expressing red cells in HU-treated patients is due to the fall in reticulocytes after HU-treatment. The percentage of reticulocytes in the HU-treated group was 5.6% compared to 11.5% in steady state sickle cell patients. The decrease in AM expressing red cells is from 2.7% in steady state to 1.6% in HU-treated patients.

In the F^+cell population, AM expression appears to be similar to the red cells as a whole, both in control subjects and sickle cell patients. This has not been previously reported. The $\%F^+$cells expressing AMs in steady state and in crisis patients were significantly higher than in control subjects and in HU-treated patients.

4.2.3. Relative adhesion molecule expression in mature red cells and in reticulocytes

Rationale

Section 4.2.2.showed that in controls, less than 1% of the total red cell population expressed AMs and about 20% of reticulocytes express AMs in both healthy controls and sickle cell patients. Section 4.2.5 showed that, this difference is likely to reflect the shedding of AMs by reticulocytes. In this section, the loss of AMs between the reticulocyte stage and the mature red cell stage is examined in more detail. This is done in two ways; firstly the %AM expression is calculated in the R^+ (reticulocyte) and R^- (mature red cell) populations and secondly the %AM expressing cells in mature red cell population is divided by the %AM expressing cells in reticulocyte population to give an 'adhesion molecule depletion ratio' (AMDR). This ratio allows a comparison of the depletion of AM^+cells in different patient and control groups.

Study design

A total of 70 blood samples were obtained from 23 normal donors (control), 23 non-transfused sickle cell patients in steady state (Sickle std), 14

non-transfused sickle cell patients in crisis (sickle cri) and 10 sickle cell patients undergoing hydroxyurea therapy (sickle HU; ranging from 0.5 to 2.0 g/day for several weeks and reaching the steady level of %HbF at the time of blood sampling). These samples are the same as in section 4.2.2. The percentages of AMs expressing cells in total mature red cells and in total reticulocytes were obtained and the AMDR of these 3 AMs were calculated. The formula is shown below.

$$\text{Adhesion molecule depletion ratio} = \frac{\%\text{ mature red cells expressing AMs}}{\%\text{ reticulocytes expressing AMs}}$$

Results

Table 4.2.3 Percentages of mature red cells (MRCs) and reticulocytes (retics) expressing AMs and the adhesion molecule depletion ratio (AMDR)

	Control (23)	SS std (23)	SS cri (14)	SS HU (10)
%CD36$^+$MRCs	0.35±0.02	0.60±0.04*	0.55±0.09*	0.43±0.08*
%CD36$^+$retics	21.58±0.87	22.23±1.59	22.19±3.24	19.91±3.67
CD36 AMDR	0.016±0.005	0.027±0.014#	0.025±0.016#	0.022±0.015#
%CD41$^+$MRCs	0.34±0.02	0.67±0.04*	0.64±0.08*	0.55±0.07*
%CD41$^+$retics	21.03±1.04	19.48±1.51	21.41±3.76	18.02±3.83
CD41 AMDR	0.016±0.005	0.034±0.016#	0.030±0.024#	0.031±0.029#
%CD49d$^+$MRCs	0.35±0.02	0.66±0.04*	0.63±0.06*	0.53±0.09*
%CD49d$^+$retics	22.19±0.90	19.65±1.52	19.63±3.14	16.92±2.58
CD49d AMDR	0.016±0.004	0.034±0.017#	0.032±0.021#	0.031±0.010#

The pattern of %CD36, %CD41 and %CD49d expressing cells is similar and is referred to as AMs. %AM$^+$reticulocytes are significantly higher than %AM$^+$mature red cells in all groups. %AM$^+$mature red cells are significantly higher in all groups of sickle cell patients than in control group (*$p<0.01$).

However, %AM$^+$reticulocytes are not significantly different in all groups of sickle cell patients and in control group. The significant higher AMDRs in all groups of patients ($^{\#}p<0.05$) over the control group, reflects a higher proportion of red cells expressing AMs after the reticulocyte stage in sickle cell disorders.

In HU-treated sickle cell patients, it appears to have similar patterns of changes between these 3 AMs. CD36 is expressed in 19.91% of reticulocytes compared to 22.23% of reticulocytes in the steady state patients, with no significant difference. CD36 is expressed in a lower proportion ($p<0.05$) of mature red cells in HU-treated patients (0.43%) than in steady state patients (0.60%). The rest of 2 AMs are also expressed in a similar trend as CD36.

Discussion

In both normal control subjects and sickle cell patients, the proportion of total mature red cells expressing AMs was significantly lower than those in total reticulocytes ($p<0.0001$). The AMDRs in the control subjects were all lower than 0.02 indicating that the majority of AM$^+$cells (over 98%) shed their AMs completely or less than 2% of the cells carry the AMs when they mature from the reticulocytes to mature red cells. AMDRs in sickle cell patients were significantly higher than those in control subjects ($p<0.05$) and less than 7% of cells remain carrying AMs beyond the reticulocyte stage. A likely explanation is that the average age of red cells is significantly shorter in sickle cell patients than in healthy controls. Thus a relatively high proportion of cells in sickle cell patients are at an younger age where AMs have not been completely shed but are RNA negative (i.e. day 3-5 after released from the bone marrow). This would mean that the proportion of AM$^+$mature red cells relative to AM$^+$reticulocytes would be greater in sickle cell patients than in control subjects, hence the AMDRs would be greater too. Therefore, the difference in AMDR between sickle cell patients and control subjects is likely to reflect the higher proportion of young red cells at the post-reticulocyte stage in sickle cell patients.

In conclusion, the findings using double-colour staining in this section show that AMs are found on reticulocytes at a similar percentages in controls and

in sickle cell patients and suggest that AMs are rapidly shed from red cells, persisting only a few days beyond the reticulocyte stage. Unanswered questions from this section include whether AMs are expressed in similar proportions in F^+reticulocytes and F^+reticulocytes in sickle cell patients and in control subjects. A further question is how hydroxyurea treatment affects this. Triple-colour staining offers the possibility of answering these questions and is described in section 4.3.

4.2.4. Relative adhesion molecule expression in F positive and F negative populations

Rationale

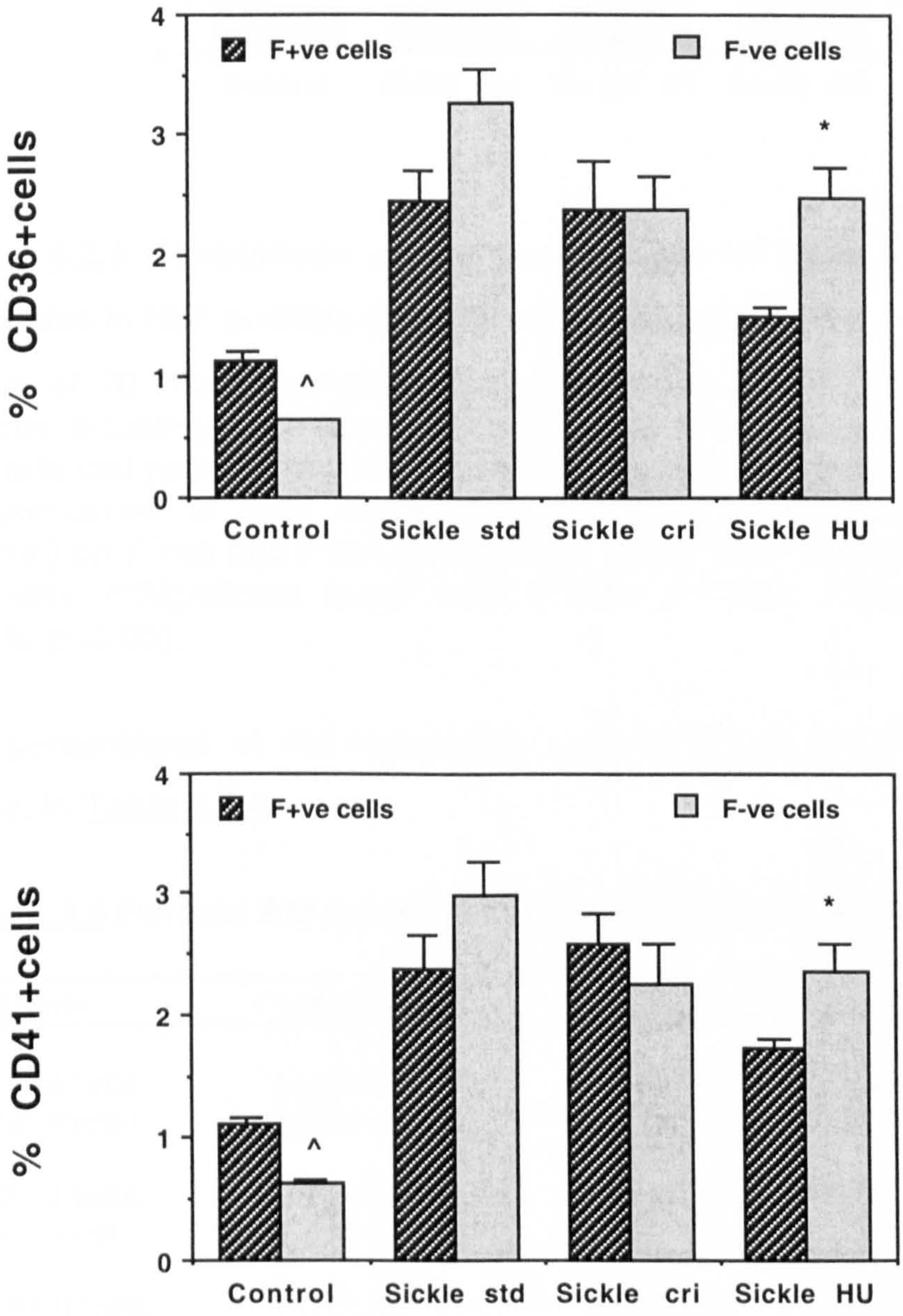
In section 4.2.2, the finding in control subjects of a higher %F^+cells expressing AMs than in the red cell population as a whole, suggests that F^+cells express more AMs than F^-cells. In this section, the difference is formally compared. The expression of AMs in F^+cells and F^-cells is potentially important because HU treatment typically increases the number of F^+cells. It is not presently known whether F^+cells express more or less AMs than F^-cells, either in healthy controls or in sickle cell patients. In order to compare the proportion of AM expressing cells in F^+cell and F^-cell populations, normal control and sickle cell blood samples were assayed using three double-colour staining flow cytometry.

Study design

A total of 70 blood samples were obtained from 23 normal donors (control), 23 non-transfused sickle cell patients in steady state (sickle std), 14 non-transfused sickle cell patients in crisis (sickle cri) and 10 sickle cell patients undergoing hydroxyurea therapy (sickle HU) (the same samples as topic 4.2.2). These were assayed for the proportion of AM expressing cells in HbF positive and HbF negative populations.

Results

The results show that in control subjects, AMs are expressed in a significantly lower % of F^-cells than F^+cells. In sickle cell patients in steady state and in crisis, the proportion of AM^+cells is about the same in F^-cell and F^+cell populations. However, in HU-treated sickle cell patients there are significantly higher AM^+cells in the F^-cell than F^+cell populations. (Figure 4.2.4.)



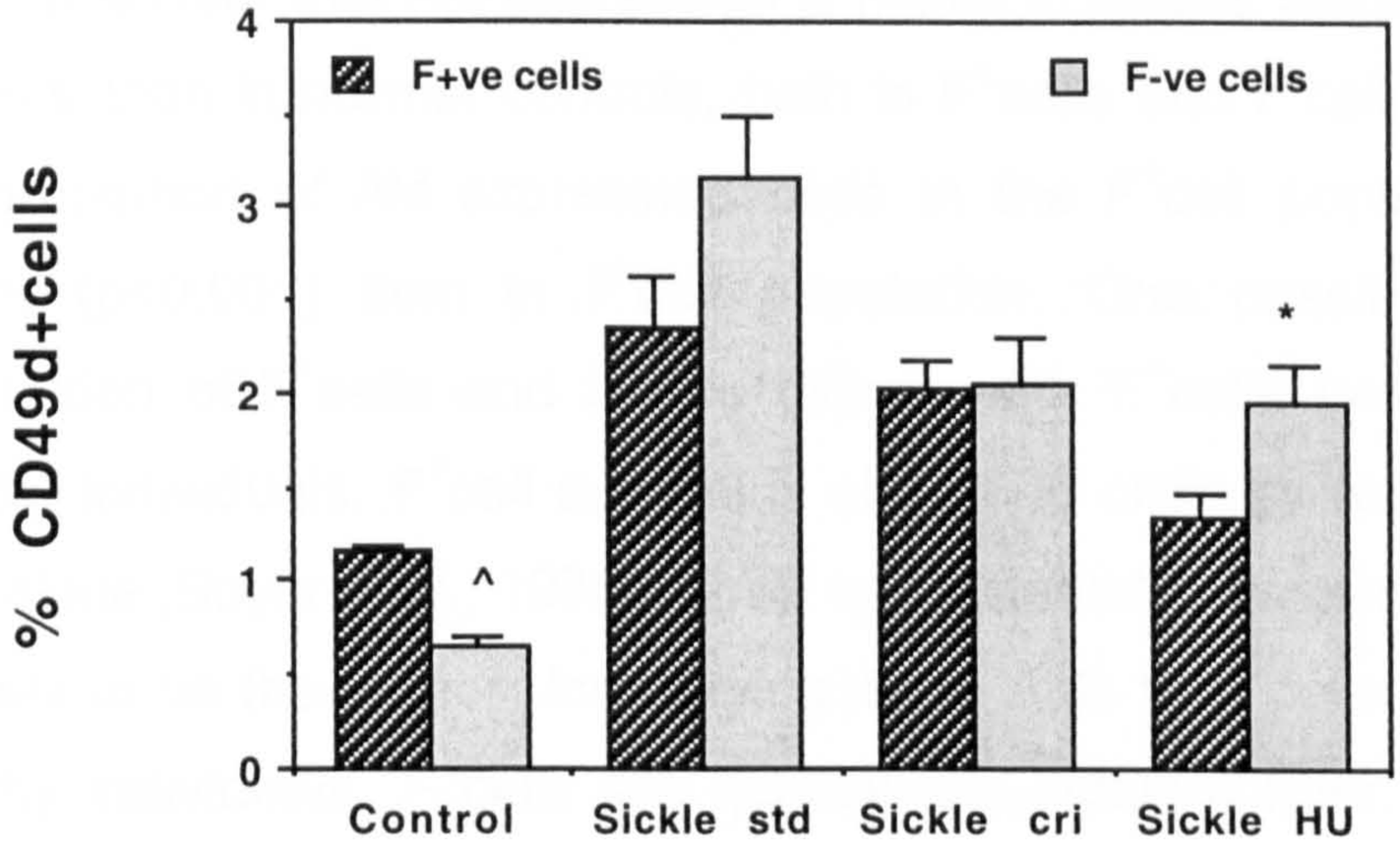


Figure 4.2.4 Comparison of the percentages of cells expressing adhesion molecules in HbF positive and HbF negative populations

A total of 70 blood samples from 23 normal donors (control), 23 sickle cell patients in steady state (sickle std), 14 sickle cell patients in crisis (sickle cri) and 10 sickle cell patients receiving hydroxyurea therapy (sickle HU) were assayed for the proportion of cells expressing CD36 (top), CD41 (middle) and CD49d (bottom) on F^+cell and F^-cell populations using three double-colour staining flow cytometry. (^Significant lower than F^+cells $p<0.001$, *Significantly higher than F^+cells $p<0.05$).

The percentages of AM expressing cells in F^+cell and F^-cell populations are shown in Table 4.2.4

Table 4.2.4 Percent AM expressing cells in F^+cell and F^-cell populations

%AM⁺cells	Control (23)	SS.std.(23)	SS.cri. (14)	SS.HU.(10)
%CD36⁺ (F^+cells)	1.14±0.05	2.46±0.26	2.32±0.31	0.90±0.12
%CD36⁺ (F^-cells)	0.64±0.03	3.26±0.28	2.42±0.21	1.75±0.37
%CD41⁺ (F^+cells)	1.13±0.04	2.39±0.26	2.53±0.21	1.43±0.23
%CD41⁺ (F^-cells)	0.62±0.03	2.98±0.27	2.09±0.27	1.97±0.43
%CD49d⁺ (F^+cells)	1.15±0.04	2.36±0.26	2.12±0.17	1.20±0.17
%CD49d⁺ (F^-cells)	0.67±0.03	3.14±0.33	2.30±0.24	2.12±0.35

Discussion

It is clear that AM expression is higher in steady state and crisis sickle cell patients than in normal controls, both in F^+cells and F^-cells. In control subjects, the proportion of AM expressing cells in the F^+cell population is significantly higher ($p<0.001$) than in F^-cell population. One possibility is that the age distribution of F^+cells and F^-cells differs, with F^+cells being more primitive. In healthy individuals, F^+cell survival is similar to ordinary red cells, which contain HbA alone (Boyer *et al.*, 1975). Their age different between F^+cells and F^-cells is unlikely to be the reason for carrying more AMs in F^+cells. It is possible that in healthy individuals, F^+cells are qualitatively different from F^-cells at the time of release from bone marrow in ways, which are unrelated to HbF content, as demonstrated here by carrying a higher proportion of AMs. Only limited data about the detailed phenotype of F^+cells as compared with F^-cells are available (Burns *et al.*, 1988). Ii antigen expression (Maniatis *et al.*, 1979) or carbonic anhydrase isoenzymes or $^{G}\gamma$ to $^{A}\gamma$ ratio (Rees *et al.* 1996) show no qualitative difference in F^+cells and F^-cells. Therefore the reason for high proportion of AM expressing cells in the F^+cell population in normal healthy individuals is unknown.

A further finding in this section is that, in sickle cell patients the proportion of AM expressing cells in the F^+cell population is relatively similar to that in the F^- cell population unlike in control subjects. Indeed, the proportion of F^-cells expressing AMs tends to be greater than the proportion of F^+cells expressing AMs, and this reaches significant level only in HU-treated patients. A possible reason may be because F^-cells survive less well than F^+cells in sickle cell disease (section 4.1.4), so that the F^-cells are younger than F^+cells. Thus, if AMs are shed or preferentially removed as a function of ageing (section 4.2.5), then it would be expected that there would be less AMs in the F^+cell population, which survives better in sickle cell disorders.

In HU-treated patients, the proportion of F^-cells expressing AMs is significantly greater than in F^+cells. A possible explanation is that F^-cells after HU treatment represent a significantly younger population than F^+cells because F^-

cells are removed more rapidly from the circulation as a result of more rapid HbS polymerisation than in F^+cells. This question is addressed directly in section 4.3 where AM expression is compared in reticulocytes and mature red cells of F^+cells and F^-cells using triple-colour staining.

This is the first study to examine AM expression in F^+cells and F^-cells. These findings provide the first evidence of differences in AM expression between F^+cells and F^-cells in healthy individuals. These are also the first findings showing that the distribution of AMs in F^+cells and F^-cells differs in sickle cell patients and healthy control subjects. The clinical significance of the findings will be discussed further at the end of the chapter. In order to learn these findings better, it is necessary to understand more about the effect of red cell ageing on AM expression. No previous studies addressing this issue are found and therefore *in vitro* sickle reticulocyte culture experiment is performed to determine the effect of red cell ageing on AM expression.

4.2.5. AM expression as a function of sickle reticulocyte maturation during *in vitro* culture

Rationale

The findings in 4.2.2 show that 1% of red cells express AMs whereas about 20% of reticulocytes express AMs. This suggests that either AMs are rapidly shed from red cells, before or shortly after the reticulocyte stage, or that AM expressing reticulocytes are preferentially removed from the circulation. Little is known about AM loss from reticulocytes. In order to address this question, measurement of AM expression as a function of time in culture of red cells taken from patients with sickle cell disease was performed. After 3 days of culture most reticulocytes typically become mature red cells, no longer containing RNA. If the shedding hypothesis is correct, after 3 days in culture there should be a reduced proportion of cells expressing AMs. On the other hand, if the clearing hypothesis is correct, the same or nearly the same proportion of cells should express AMs as at the start of the culture. The double-colour staining developed in chapter 3

allowed us to examine whether AM expression changes at a faster or slower rate than the disappearance of reticulocytes.

Study design

A total of 8 blood samples from sickle cell patients in steady state were collected in EDTA vacutainers. The blood samples were cultured in vitro for 15 days (section 2.5.6). The samples were assayed for proportions of AM expressing cells and reticulocytes on days 0 (within the first hour of culture), 1, 2, 3, 4, 5, 10 and 15 respectively, using three double-colour staining flow cytometry (section 3.4.2).

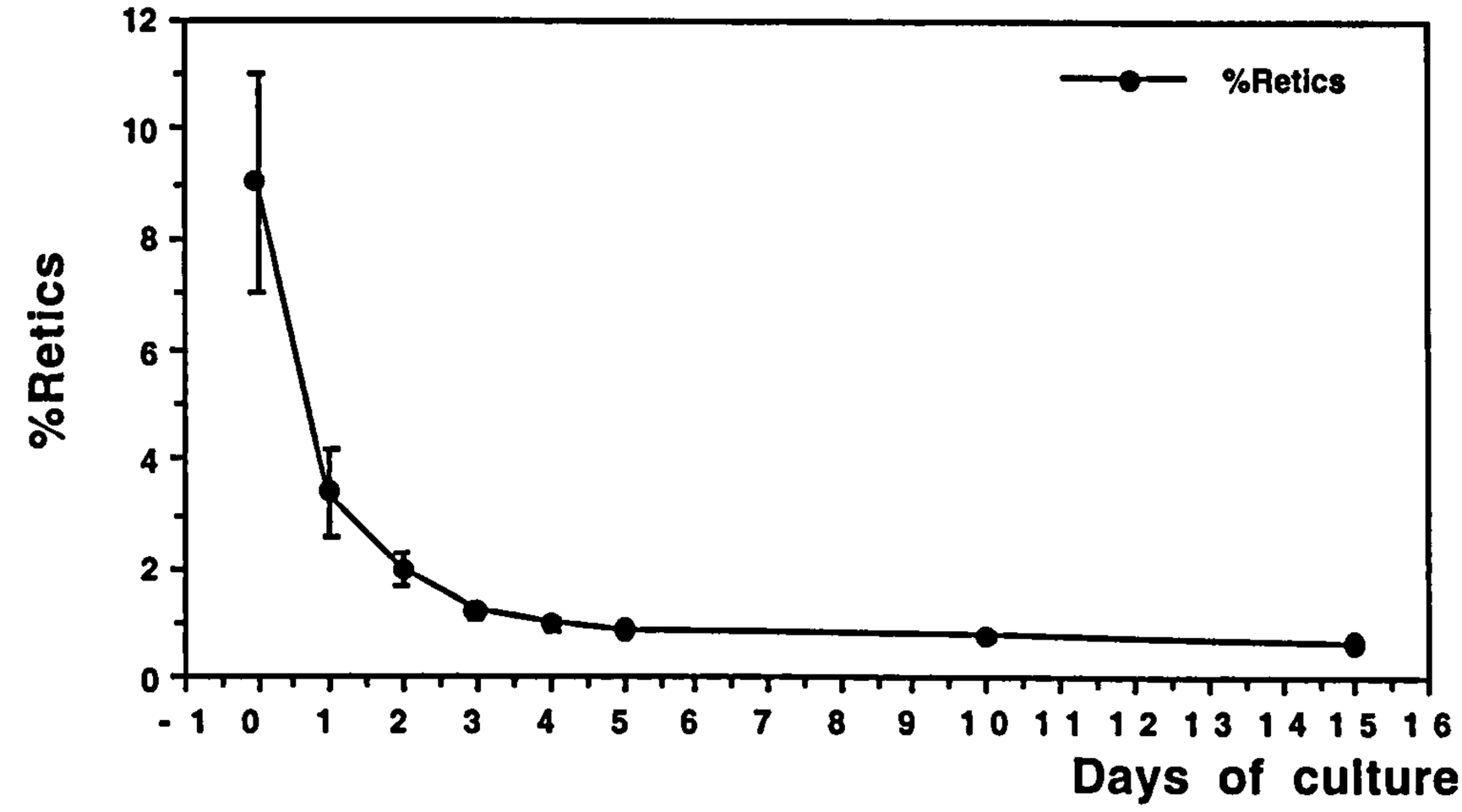
Results

Mean percentages of reticulocytes (n=8) on days 0, 1, 2, 3, 4, 5, 10 and 15 of culture were 9.0±2.0, 3.4±0.8, 2.0±0.3, 1.2±0.1, 1.0±0.1, 0.9±0.1, 0.8±0.1 and 0.7±0.1% respectively. Each consecutive day showed a significant reduction ($p<0.05$) in the mean % of reticulocytes compared to the previous day for the first 3 days, whereas the reductions between day 3 and day 4 through day 15 were not significantly different.

Mean %$CD36^+$cells from the above samples were 1.5±0.08, 1.2±0.06, 1.0±0.06, 0.8±0.06, 0.7±0.06, 0.6±0.04, 0.6±0.04 and 0.5±0.04% on each day respectively. Reductions on consecutive days were significant ($p<0.01$) when compared to the value of the previous day, except for reductions between day 5 and day 10 and between day 10 and day 15 where the reductions were not significantly different.

Mean %$CD41^+$cells from the above samples were 1.6±0.10, 1.2±0.07, 1.1±0.05, 1.0±0.03, 0.9±0.04, 0.8±0.04, 0.7±0.03 and 0.7±0.04% respectively. The reductions were as same as of %$CD36^+$cells above.

Mean %$CD49d^+$cells of the same samples as above were 1.7±0.14, 1.4±0.10, 1.2±0.07, 1.1±0.05, 1.0±0.03, 0.8±0.04, 0.8±0.04 and 0.7±0.03% on each day respectively. The reductions were as same as of %$CD36^+$cells above. (Figure 4.2.5)



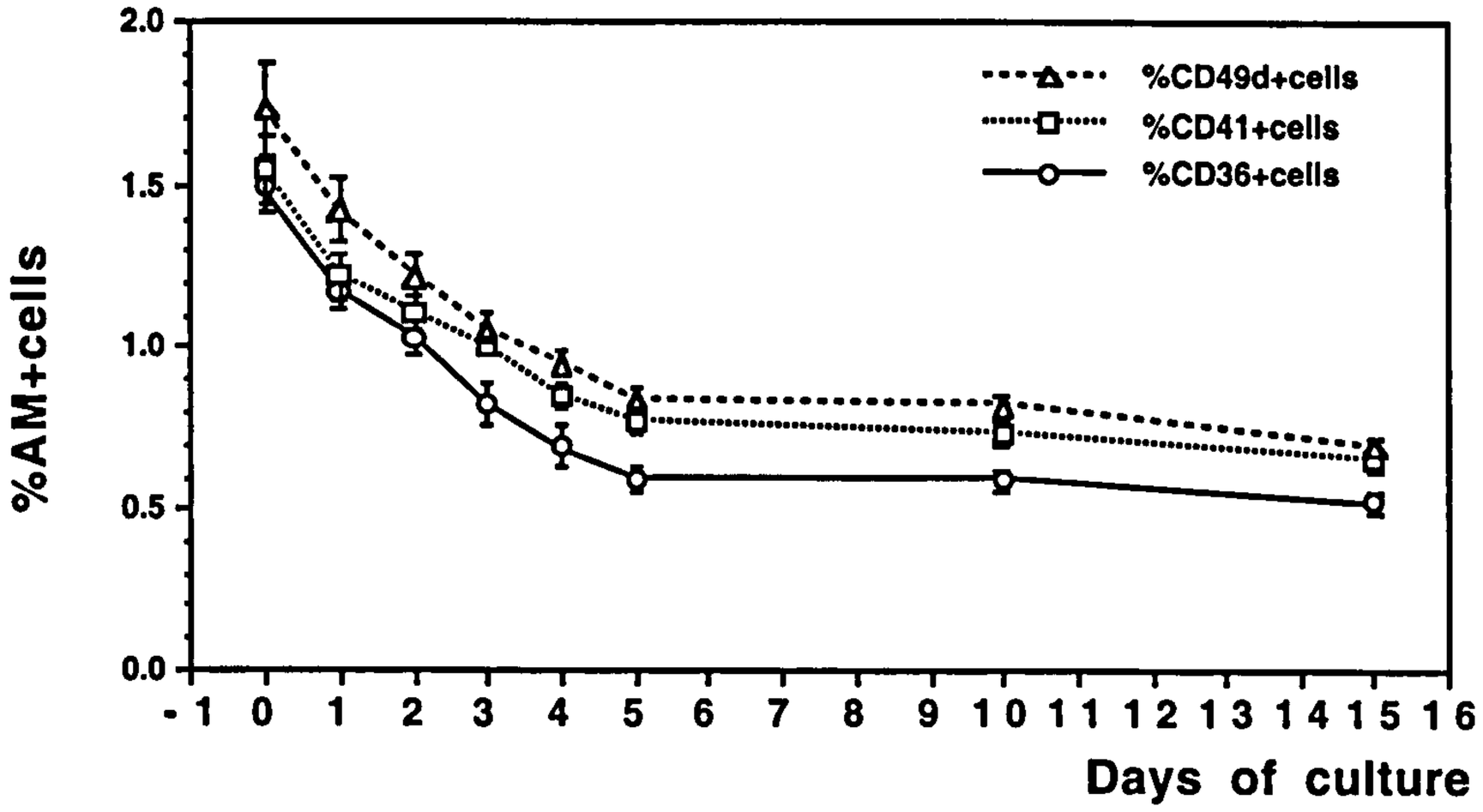


Figure 4.2.5 Reductions in percentages of reticulocytes and AM expressing cells in culture *in vitro*

A total of 8 sickle cell blood samples were cultured in RPMI-1640 supplemented with CPD, FCS, penicillin and streptomycin at 37°C, in 5% CO_2 atmosphere for 15 days. Percentages of reticulocytes and AM (CD36, CD41 and CD49d) expressing cells were determined by three double-colour staining flow cytometry. Percentages of reticulocytes were significantly lower than the value of the day before for the first 3 days ($p<0.05$). Percentages of all 3 AM expressing cells were significantly lower than the value of the day before for the first 5 days ($p<0.01$).

Discussion

The results clearly show a reduction in the % reticulocytes over 3 days in culture. Levels fell from 9% on day 0 to approximately 1% by day 3, indicating that nearly all sickle reticulocytes became mature red cells within 3 days of culture. This finding is consistent with the reticulocyte maturation rate of about 3 days (McKenzie, 1996).

The results also show that proportions of all 3 AM expressing cells fell within the first few days in culture, reaching baseline levels at 5 days. Thereafter the levels do not fall further. It is likely that these levels represent the limit of detection of the assay.

These findings are important in the interpretation of results in section 4.2.2 and 4.2.3 and also confirm the findings of other investigators (Swerlick et al. 1993; Gee *et al*. 1995; Styles *et al*. 1997). The implications are that the small % of AM^+mature red cells seen both in healthy subjects and in sickle cell patients primarily reflects shedding of AMs from reticulocytes within 5 days of release from the bone marrow. Therefore changes in the % of cells expressing AMs could occur as a result of changes in the mean age of the cell population being studied. A younger population of reticulocytes would be expected to express more AMs than an older population. Therefore HU, which can prolong red cell survival by increasing HbF, decreases the proportion of reticulocytes and AM expressing cells, but might also be expected to allow a greater number of F^+reticulocytes to age into red cells. The results in Table 4.2.3 show a small decrease (with no significant difference) in the % of reticulocytes expressing AMs in HU-treated patients compared to non-HU-treated patients (from about 20% to 18%) which is consistent with the cells ageing because of reduction in haemolysis. This finding differs from Styles *et al*. 1997, who found a significant decrease in the % of reticulocyte expressing AMs. However the patients in that study were all children (aged between 2-15 years) and mainly males (7 male and 1 female), while in this thesis a mixture of both children and adult (aged between 12-57 years) and more balanced gender (6 male and 4 female) were studied. These factors may give a different outcomes

4.3. CO-DISTRIBUTION OF ADHESION MOLECULES AND FOETAL HAEMOGLOBIN

4.3.1. Background

Triple-colour staining flow cytometry for determination of the co-distribution of AMs (CD36, CD41 and CD49d), HbF (F^+cells) and RNA (reticulocytes) was employed (section 3.4.3) in this section. Using this method, the red cells are divided into many sub-populations, which can be grouped into 3 groups. Single variables consist of AM^+, AM^-, F^+, F^-, R^+ and R^- subpopulations. Double variables consist of AM^+F^+, AM^+F^-, AM^-F^+, AM^-F^-, AM^+R^+, AM^+R^-, AM^-R^+, AM^-R^-, F^+R^+, F^+R^-, F^-R^+ and F^-R^-. Triple variables consist of $AM^+F^+R^+$, $AM^+F^+R^-$, $AM^+F^-R^+$, $AM^-F^+R^+$, $AM^+F^-R^-$, $AM^-F^-R^+$, $AM^-F^+R^-$ and $AM^-F^-R^-$. Questions that can in principle be answered with triple-colour analysis are as follows.

Does the proportion of F^+cells expressing AMs differ from the proportion of F^-cells expressing AMs at the reticulocyte (R^+) stage? This is potentially important because if recently released F^+reticulocytes from the bone marrow are qualitatively different and express more AMs than F^-reticulocytes, then the beneficial effect of HU treatment on increasing F^+cells could be counteracted by an increase in AMs on such cells.

To what extent can the presence of HbF in sickle red cells affect the proportion of cells with residual AM expression beyond the reticulocyte stage (R^- or mature red cell stage) in sickle cell disorders?

How does HU treatment affect the distribution of AMs on F^+cells and F^-cells at the reticulocyte stage? If HU treatment decreases AM expression on F^+reticulocytes then there could be a double benefit on red cell survival.

Does HU treatment decrease the AM expressing cells simply by decreasing the reticulocyte numbers or does the HU-treatment reduce % of AMs expressing reticulocytes?

Does the effect of HU on HbF and AM expression have a similar kinetic or does the decrease in AM expression begin first?

4.3.2. Comparison of single variables obtained from triple-colour and double-colour staining

Rationale

In section 4.2, a number of single variables were measured using double-colour staining. In this section the same variables were investigated using the triple-colour staining procedure. If the analysis is valid, then the proportions of cells in each category should be approximately the same as using double-colour staining procedure. The patient groups studied here are similar but using different groups of patients and control subjects. Therefore the values obtained will not be identical. However in section 3.4.4 and 3.4.7, these values were comparable using double- and triple-colour staining.

Study design

Blood samples from 30 normal control subjects (Control), 35 non-transfused sickle cell patients in steady state (SS.std.), 22 non-transfused sickle cell patients in crisis (SS.cri.) and 10 sickle cell patients undergoing HU therapy (SS.HU.) at University College London Hospitals (UCLH), London, UK were taken into either EDTA, citrate, heparin or CTAD. Plasma was removed then Alsever : PBS (1:1) was added to the red cells to give approximately 45-50% haematocrit and the suspension was kept at 4°C. The samples were assayed using triple-colour staining flow cytometry (section 3.4.3) within 3 days of collection. %HbF was assayed by HPLC (section 2.5.2).

Results

Percentages of red cell sub-populations in control subjects (n=30), SS.std. (n=35) SS.cri. (22) and SS.HU. (n=10) are shown in Table 4.3.2.1.

Table 4.3.2 Comparison of patient data and single variables

Variable	Control (30)	SS.std.(35)	SS.cri. (22)	SS.HU.(10)
Age (yr.)	N.D.	25.69±2.32	30.09±2.12	35.20±4.88
Sex (m:f)	N.D.	15:20	10:12	6:4
RBCs ($10^6/\mu l$)	4.70±0.074	2.87±0.12^	2.78±0.10^	2.69±0.14^
Hb (g/dl)	13.86±0.23	8.02±0.19^	7.77±0.29^	8.64±0.44^
HbF (%)	0.78±0.10	6.43±0.69*	5.10±0.51*	22.18±1.76*
F^+cells %	4.22±0.21	29.96±2.53*	24.43±2.13*	59.38±3.41*§
number	199.23±10.78	822.05±63.41*	660.94±55.71*	1584.07±102.35*§
Retics %	1.57±0.05	11.14±0.68*	9.74±0.74*	7.37±0.89*#
number	74.02±2.92	319.00±20.64*	265.81±20.98*	196.68±26.83*#
$CD36^+$cells %	0.66±0.02	3.05±0.29*	2.66±0.24*	1.96±0.21*#
number	31.02±1.32	92.35±11.44*	73.73±6.78*	52.92±6.86*
$CD41^+$cells %	0.65±0.03	2.88±0.30*	2.40±0.19*	2.00±0.17*#
number	30.97±1.46	86.87±11.52*	66.72±5.83*	54.14±5.94*
$CD49d^+$cells %	0.69±0.03	3.04±0.32*	2.74±0.29*	2.08±0.20*#
number	32.71±1.46	92.29±12.31*	75.73±7.49*	56.16±6.83*

All parameters are presented as mean±SEM, SS = sickle cell patients, std = in steady state, cri = in crisis, HU = on hydroxyurea treatment, N.D. = No data, m:f = male : female, Retics = reticulocytes
^ significantly lower than control $p<0.001$, * significantly higher than control $p<0.001$, § significantly higher than SS.std. $p<0.001$, # significantly lower than SS.std. $p<0.05$,

Discussion

In these groups of controls and patients, the findings broadly are similar to those in section 4.2. As expected, the RBC and Hb are lower in sickle patients than in controls. The number and % of reticulocytes are higher in sickle cell patients than controls (*$p<0.001$) and lower in HU-treated patients than in steady state patients (#$p<0.05$). The numbers and % of F^+cells are higher in sickle cell patients in steady state than in controls (*$p<0.001$) and higher in HU-treated patients than in steady state patients ($p<0.001$). The numbers and % of AM expressing cells is higher ($p<0.001$) in sickle cell patients in steady state than in

controls and lower in HU-treated patients than in steady state patients ($p<0.05$). These findings agree with section 4.2.

4.3.3. Comparison of double variables obtained from triple-colour analysis and double-colour analysis

Study design

In this section, the co-distributions of HbF in reticulocytes and mature red cells; of RNA in F^+cells and F^-cells; of AMs in reticulocytes and mature red cells and of AMs in F^+cells and F^-cells were measured using triple-colour staining. The results should broadly agree with those in sections 4.1 and 4.2, if the triple-colour staining is valid. In addition to sections 4.1 and 4.2, absolute numbers of these populations as well as proportions are determined.

Results and discussion

HbF expression in reticulocyte (RNA+) and mature red cell (RNA-) populations

In section 4.1.3, the proportions of F^+reticulocytes and F^+cells were compared. In this section, absolute numbers are measured as well as proportions. Additionally F^+cells are compared, specifically in reticulocyte (R^+) and mature red cell (R^-) populations, rather than in the total red cell population as in section 4.1.3.

Table 4.3.3.1 HbF expression in reticulocyte (R+) and mature red cell (R-) populations *(to be compared with 4.1.3)*

Parameter	Control (30)	SS.std.(35)	SS.cri. (22)	SS.HU.(10)
F^+reticulocyte %	4.36±0.20	12.88±0.88*	13.13±1.37*	26.25±2.43*#
number	3.21±0.20	39.42±3.13*	34.35±3.99*	48.54±5.95*#
F^+mature red cell %	4.23±0.21	32.19±2.78* §	25.70±2.27*§	62.22±3.71*#§
number	195.90±10.70	789.40±60.37*§	626.59±53.06*§	1535.98±100.26*#§
F^+ enrichment ratio	1.00±0.04	2.51±0.15*	2.62±0.26*	2.12±0.15*

All parameters are presented as mean±SEM, number = $x10^3$ / cu.mm., SS. = Sickle cell patients, std. = in steady state, cri. = in crisis state, HU. = on hydroxyurea, F = foetal haemoglobin (HbF), R = RNA (reticulocytes)
* significantly higher than control $p<0.05$, # significantly higher than SS.std. $p<0.05$, § significantly higher than F^+reticulocytes $p<0.001$,

These findings show that in control subjects, the proportion of F^+cells in reticulocytes and mature red cells is similar. This means that the distribution of F^+cells and F^-cells in healthy controls is likely to be similar. This has not been shown previously. In section 4.1.3, the % of F^+reticulocytes was compared with F^+cells in the total red cell population, but the analysis in this section is more precise because it compares reticulocytes (R^+) with mature red cells (R^-) rather than the total red cell population.

As expected, the number of F^+mature red cells is greater than the number of F^+reticulocytes, because the number of red cells is greater than the number of reticulocytes. In all groups of sickle cell patients, the proportion of F^+mature red cells exceeds that of F^+reticulocytes by about two fold, as reflected by the ER over 2.00. These ERs are in agreement with section 4.1.4, which was obtained from double-colour staining. (Table 4.3.3.1)

Reticulocyte distribution in F^+ and F^- populations

Table 4.3.3.2 Reticulocyte (RNA) expression in F^+cell and F^-cell populations

Parameter	Control (30)	SS.std.(35)	SS.cri. (22)	SS.HU.(10)
R^+ (F^+cells) %	1.66±0.11	5.16±0.40*	5.21±0.44*	3.10±0.38*
number	3.21±0.20	39.42±3.13*	34.35±3.99*	48.54±5.95*#
R^+ (F^-cells) %	1.57±0.06	15.56±1.03*§	11.36±0.91*§	14.56±2.28*§
number	70.81±2.81§	278.74±19.22*§	231.52±19.91*§	148.14±22.27*§^

All parameters are presented as mean±SEM, number = $x10^3$ / cu.mm., SS. = Sickle cell patients, std. = in steady state, cri. = in crisis state, HU. = on hydroxyurea, F = foetal haemoglobin (HbF), R = RNA (reticulocytes), R^+(F^+cells) = % of F^+cells containing RNA or F^+retics in total F^+cells, R^+(F^-cells) = % of F^- cells containing RNA or F^-retics in total F^-cells, retics = rteticulocytes.
* significantly higher than control $p<0.05$, § significantly higher than F^+cells $p<0.01$, # significantly higher than SS.std. $p<0.05$, ^ significant lower than SS.std. $p<0.01$

These findings show that F^+cells and F^-cells contain the same proportion of reticulocytes in healthy controls and that therefore the age distribution up to the reticulocyte stage is similar in F^+cell and F^-cell populations. Thus the observed difference in the proportions of AMs in F^+cell and F^-cell populations seen in section 4.2.4 is unlikely to be due to the age differences of the cells. This suggests that in healthy controls, F^+cells contain more AMs than F^-cells. This question can only be answered directly using triple-colour analysis for co-distribution of F^+, R^+ and AM^+, which is performed, in section 4.3.4.

In all sickle cell patient groups, the %reticulocyte is lower ($p<0.001$) in F^+cell than in F^-cell populations, consistent with a higher % of cells maturing beyond the reticulocyte stage in F^+cell than in F^-cell populations. The data comparing the number and %reticulocytes within F^+cell and F^-cell populations has not been presented before. (Table 4.3.3.2)

When absolute numbers of F^+cell and F^-cell populations are examined, in patients on HU treatment, the number of R^+F^+cells increases compared with steady state patients ($p<0.01$), whereas the number of R^+F^-cells decreases

($p<0.01$). This is consistent with an increase in release of F^+reticulocytes from the bone marrow following HU treatment.

Distribution of AM expressing cells in reticulocyte and in mature red cell populations

In this section the distribution of AMs in reticulocytes and mature red cells is compared. The results in this section are directly comparable with those in 4.2.3; the difference being that the data were obtained from triple-colour staining rather than double-colour staining. The patient and control samples were independent of section 4.2.3, but the sample size was larger. Despite these differences, the results are essentially similar to those of section 4.2.3.

Table 4.3.3.3 Distribution of AM expressing cells in reticulocyte and mature red cell populations *(to be compared with 4.2.3)*

Parameter	Control (30)	SS.std.(35)	SS.cri. (22)	SS.HU.(10)
CD36⁺mature red cells %	0.32±0.02	0.76±0.15*	0.64±0.07*	0.64±0.10*
number	15.03±0.99	21.19±4.95	15.88±1.90	15.88±2.66
CD36⁺reticulocytes %	22.63±0.98§	23.09±1.65§	22.31±1.93§	19.23±1.40§
number	16.17±0.82	71.21±7.46*§	57.86±5.84*§	37.04±5.05*§
CD36⁺ AMDR	0.015±0.001	0.034±0.003*	0.032±0.004*	0.035±0.006*
CD41⁺mature red cells %	0.31±0.020	0.85±0.17*	0.71±0.07*	0.77±0.07*
number	14.69±0.99	23.08±5.20	17.20±1.36	19.34±1.98
CD41⁺reticulocytes %	22.14±1.02§	20.52±1.44§	20.20±2.31§	18.17±1.33§
number	16.27±0.98	65.49±8.39*§	49.52±5.65*§	37.18±5.23*§
CD41⁺ AMDR	0.015±0.001	0.044±0.005*	0.047±0.006*	0.046±0.006*
CD49d⁺mature red cells %	0.33±0.02	0.86±0.17*	0.77±0.12*	0.81±0.09*
number	15.25±1.06	23.23±5.07	18.57±2.55	20.16±2.46
CD49d⁺reticulocytes %	23.43±1.06§	21.43±1.65§	21.64±1.96§	18.45±1.43§
number	17.44±0.91	69.07±8.35*§	55.42±5.33*§	36.47±5.70*§
CD49d⁺ AMDR	0.014±0.001	0.042±0.004*	0.041±0.006*	0.047±0.007*

All parameters are presented as mean±SEM, number = $x10^3$ / cu.mm., SS. = Sickle cell patients, std. = in steady state, cri. = in crisis state, HU. = on hydroxyurea, F = foetal haemoglobin (HbF), R = RNA (reticulocytes),
* significantly higher than control $p<0.05$, § significantly higher than mature red cells $p<0.001$,

Distribution of AM expressing in F^+cell and F^-cell populations

In this section, the distribution of AMs in F^+cells and F^-cells is compared. The results in section are directly comparable with those in 4.2.4; the difference being that the data were obtained from triple-colour staining rather than double-colour staining. The patient and control samples were independent of section 4.2.4 but the sample size was larger. Despite these differences, the results are essentially similar to those of section 4.2.4.

Table 4.3.3.4 Distribution of AM expressing cells in F^+cell and F^-cell populations *(to be compared with 4.2.4)*

Parameter	Control (30)	SS.std.(35)	SS.cri. (22)	SS.HU.(10)
$CD36^+$ (F^+cells) %	1.35±0.13	2.09±0.23*	1.89±0.24*	0.76±0.08#
number	2.56±0.22	15.44±1.44*	11.81±1.83*	12.87±1.94*
$CD36^+$ (F^-cells) %	0.63±0.02^	3.64±0.35*	2.97±0.30*	4.07±0.63*§
number	28.62±1.27§	76.73±10.73*§	61.92±6.22*§	40.31±6.12*§#
$CD41^+$ (F^+cells) %	1.29±0.11	2.07±0.24*	2.09±0.23*	0.98±0.09#
number	2.50±0.22	15.39±1.75*	12.76±1.51*	15.60±1.75*
$CD41^+$ (F^-cells) %	0.62±0.03^	3.43±0.36*	2.58±0.25*	3.93±0.54*§
number	28.44±1.36§	71.47±10.40*§	53.97±5.71*§	38.93±5.48*§#
$CD49d^+$ (F^+cells) %	1.33±0.12	2.13±0.29*	1.86±0.16*	0.97±0.09#
number	2.49±0.20	16.58±2.52*	11.34±1.05*	15.79±2.23*
$CD49d^+$ (F^-cells) %	0.67±0.03^	3.65±0.37*	3.04±0.36*	3.95±0.57*§
number	29.91±1.36§	76.24±10.83*§	62.65±6.51*§	40.83±6.00*§#

All parameters are presented as mean ± SEM, number = $x10^3$ / cu.mm., SS. = Sickle cell patients, std. = in steady state, cri. = in crisis state, HU. = on hydroxyurea, F = foetal haemoglobin (HbF), R = RNA (reticulocytes)

* significantly higher than control $p<0.05$, § significantly higher than F^+cells $p<0.05$, ^ significantly lower than F^+cells $p<0.01$, # significantly lower than SS.std. $p<0.05$,

Discussion

The percentages of F^+cells, F^+mature red cells and F^+reticulocytes in sickle cell patients both with hydroxyurea therapy (SS.HU) and without (SS.std. and SS.cri.) are significantly higher than in control subjects and these proportions in SS.HU. are also significantly higher than SS without HU as expected. The %reticulocytes are also increased in sickle cell patient groups compared to the control group, but in SS.HU. the %reticulocytes were significantly lower than in SS without HU. These findings show the effectiveness of hydroxyurea in increasing the production of HbF and its ability to suppress bone marrow, which is shown by a reduction in the %reticulocytes.

The $\%AM^+$cells in SS.HU. are significantly lower than in SS without HU (SS.std. and SS.cri), but in all sickle cell patient groups $\%AM^+$cells were significantly higher than in the control group. This reconfirms the previous findings of increased AM^+cells in total red cells of sickle cell patients. However, hydroxyurea treatment appeared to reduce these proportions. This may be the explanation of improvement of the clinical symptoms that occurs even before the increasing in HbF levels.

These findings are in agreement with section 4.2 where double-colour staining was used. This indicates that either double- or triple-colour staining gives roughly the same results for both single and double variables.

4.4. INFLUENCE OF FOETAL HAEMOGLOBIN ON ADHESION MOLECULE EXPRESSION

Rationale

The advantage of triple-colour staining is the ability to determine triple variables such as $AM^+F^+R^+$, $AM^+F^+R^-$, $AM^+F^-R^+$, $AM^-F^+R^+$, $AM^+F^-R^-$, $AM^-F^+R^-$, $AM^-F^-R^+$ and $AM^-F^-R^-$ subpopulations. Mature red cells are defined as R^-cells or red cells without RNA, while reticulocytes are defined as R^+cells or red cell with RNA. These variables may provide a greater understanding of the contribution of HbF, AMs and ageing status on the survival of red cells in sickle cell disorders especially when the patients has been treated with hydroxyurea.

Study design

In this section, triple-variables are obtained from triple-colour staining in order to compare these variables in control subjects and sickle cell patients in steady state, in crisis and on HU treatment. The patient groups and samples are identical to those in section 4.3.2.

Results and discussion

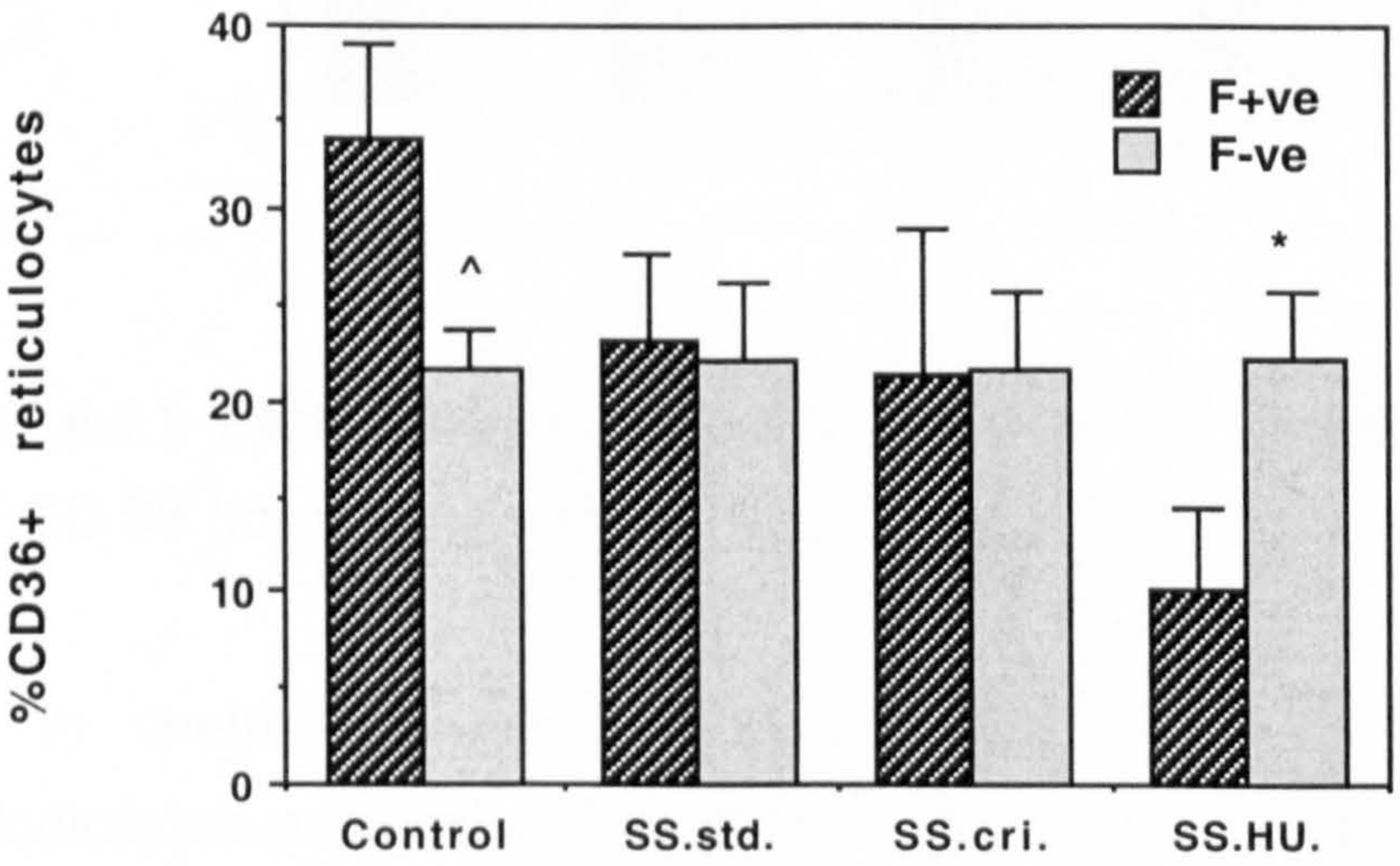
F^+reticulocytes and F^-reticulocytes expressing AMs

The results are shown in absolute numbers in Table 4.4.1.and in % in Figure 4.4.1.

Table 4.4.1 Absolute numbers of F^+reticulocyte and F^-reticulocyte expressing AMs

Parameter	Control (30)	SS.std.(35)	SS.cri. (22)	SS.HU.(10)
AM expressing F+ve reticulocytes				
$CD36^+F^+$Retics	1.08±0.09	9.37±1.02*	7.59±1.52*	4.83±1.29*#
$CD41^+F^+$Retics	1.05±0.09	8.12±0.91*	6.77±1.06*	5.47±1.30*#
$CD49d^+F^+$Retics	1.04±0.08	8.70±1.22*	6.33±0.90*	5.33±1.26*#
AM expressing F-ve reticulocytes				
$CD36^+F^-$Retics	15.09±0.78	58.44±6.22*	50.26±5.31*	32.47±5.27*#
$CD41^+F^-$Retics	15.24±0.95	51.61±6.09*	42.74±5.31*	29.73±5.13*#
$CD49d^+F^-$Retics	16.40±0.89	56.98±6.81*	49.09±5.19*	31.13±5.70*#
AM negative F+ve reticulocytes				
$CD36^-F^+$Retics	2.11±0.14	29.72±2.53*	27.51±3.96*	43.11±6.66*§
$CD41^-F^+$Retics	2.19±0.15	30.69±2.63*	27.22±3.50*	45.28±5.22*§
$CD49d^-F^+$Retics	2.17±0.15	30.07±2.77*	27.62±3.88*	41.60±5.66*§
AM negative F-ve reticulocytes				
$CD36^-F^-$Retics	54.17±2.44	206.19±14.03*	180.92±16.25*	113.73±15.24*#
$CD41^-F^-$Retics	55.82±2.60	212.32±13.94*	183.12±17.45*	117.51±19.09*#
$CD49d^-F^-$Retics	55.71±2.53	215.60±13.18*	185.75±18.65*	119.84±20.21*#

Absolute number at $x10^3$ cells/µl, SS. = Sickle cell patients, std. = in steady state, cri. = in crisis state, HU. = on hydroxyurea, F = HbF (foetal haemoglobin), R = RNA (reticulocytes), Retics = reticulocytes
* significantly higher than control $p<0.05$, § significantly higher than SS.std. $p<0.05$, ^ significantly lower than control $p<0.01$, # significantly lower than SS.std. $p<0.05$,



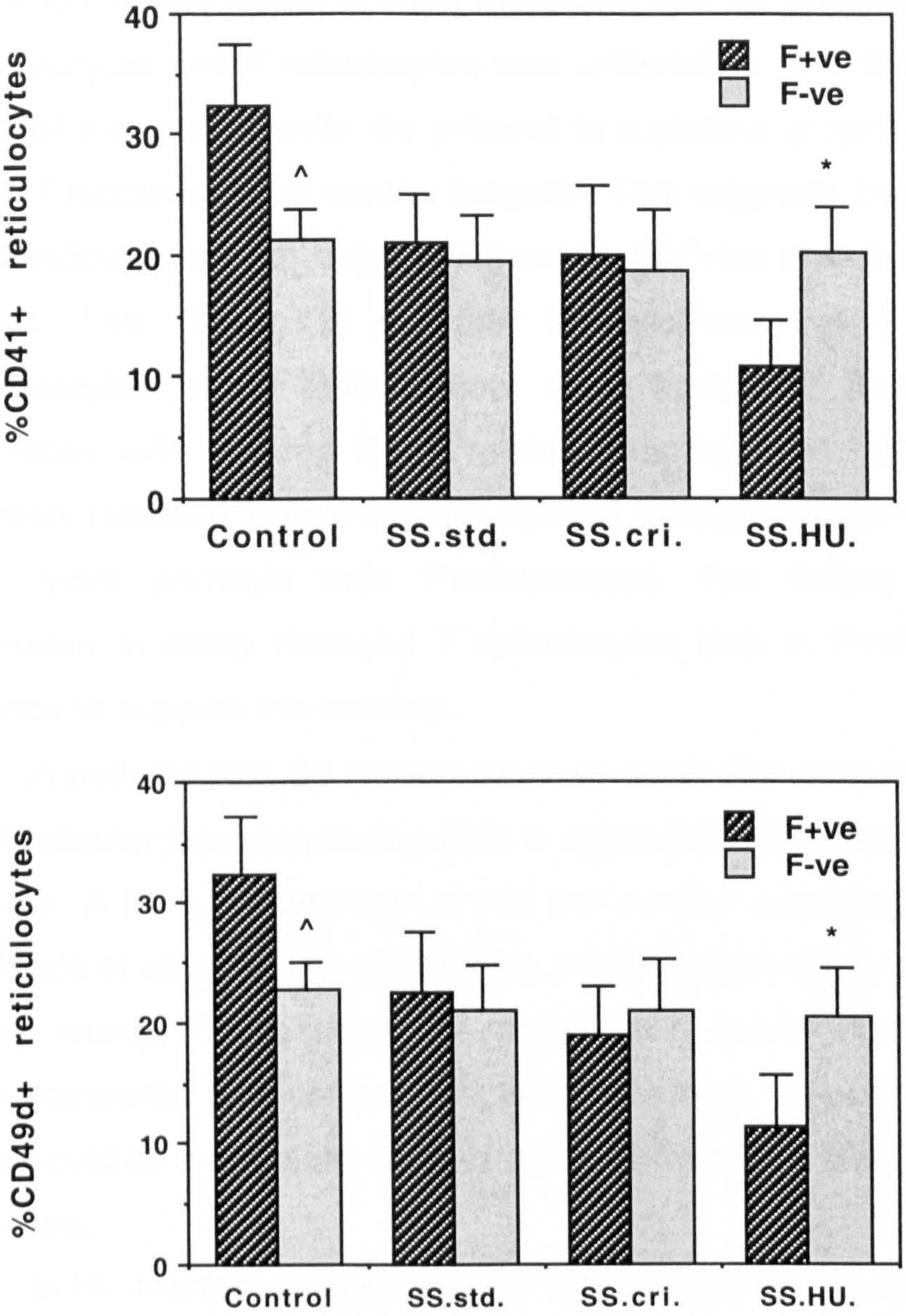


Figure 4.4.1 Percentages of F^+reticulocytes and F^-reticulocytes expressing AMs CD 36 (top), CD41 (middle) and CD49d (bottom).

In control subjects, there are about 15 times more number of F^+reticulocytes expressing AMs than number of F^-reticulocytes expressing AMs. This is compatible with the greater number of F^-reticulocytes about 23 times (70.81 $x10^3$/cu.mm.) than F^+reticulocytes (3.21 $x10^3$/cu.mm.) previously shown in

Table 4.3.3.2, suggesting an enrichment of AM expression in F^+reticulocytes relative to F^-reticulocytes. In order to confirm this, an analysis of the $\%AM^+$cells in F^+reticulocytes and F^-reticulocytes was undertaken and this is shown in Figure 4.4.1. AM expressing cells are present in a greater proportion in F^+reticulocytes than in F^-reticulocytes in control subjects. This suggests that F^+reticulocytes differ from F^-reticulocytes not only in expressing HbF but also in retaining a greater % of AMs. This finding is the first demonstration of a difference between F^+reticulocytes and F^-reticulocytes, apart from HbF content, and would be compatible with the idea that F^+reticulocytes represent a subpopulation which has been released from the bone marrow having undergone less divisions and being more primitive than F^-reticulocytes. The finding here of more AM expression in newly released F^+reticulocytes than in F^-reticulocytes is the first evidence to support this concept.

In patients with SS (steady state or crisis), the proportion of F^+reticulocytes and F^-reticulocytes expressing AMs is approximately the same, unlike in control subjects. A likely explanation is that the survival advantage of cells containing HbF leads to an older age distribution in F^+reticulocytes related to F^-reticulocytes. In other words, F^-cells are being preferentially removed even at the reticulocyte stage compared to F^+cells. In HU treated patients it would be expected that this effect would be even more marked if the HbF content and % of F^+reticulocytes is increased.

In HU treated patients, the $\%F^+$reticulocytes expressing AMs is significantly less than the $\%F^-$reticulocytes expressing AMs, consistent with the idea that a higher proportion of F^+cells survive long enough to lose more of their AMs than F^- cells which survive shorter in HU treated patients. In section 4.5, evidence will be provided that there is indeed a higher HbF content in HU treated patients, further supporting this explanation. In Figure 4.2.5, AMs fall to undetectable levels after 5 days in culture. In the same culture, reticulocytes fell more rapidly, reaching undetectable levels by day 3. The findings in Figure 4.4.1 therefore suggest that if the survival difference in F^+reticulocytes and F^-reticulocytes explains the decreased $\%F^+$reticulocytes expressing AMs following HU treatment, then many

cells would be removed from the F⁻reticulocyte population within 3 days of release from the bone marrow. In section 4.5 this question is addressed again, in paired analysis of the same patients before and after HU treatment.

Overall in this section, it is clear that AM expression in F⁺reticulocyte differs from F⁻reticulocyte populations. However control subjects, SS and HU-treated SS patients differ in AM expression. It is hypothesised that the expression of AMs in a higher proportion of F⁺reticulocytes than F⁻reticulocytes in the controls is a reflection of an accelerated release of F⁺reticulocytes from the bone marrow. Additionally, lowering of the %AM⁺F⁺reticulocytes in SS patients and further lowering of this % in HU-treated SS patients reflects the relative survival advantage of high HbF content in F⁺cells in patient with SS and thus because of the older age distribution of F⁺cells in the SS patients that leads F⁺cells to have more time to shed more AMs.

F⁺mature red cells and F⁻mature red cells expressing AMs

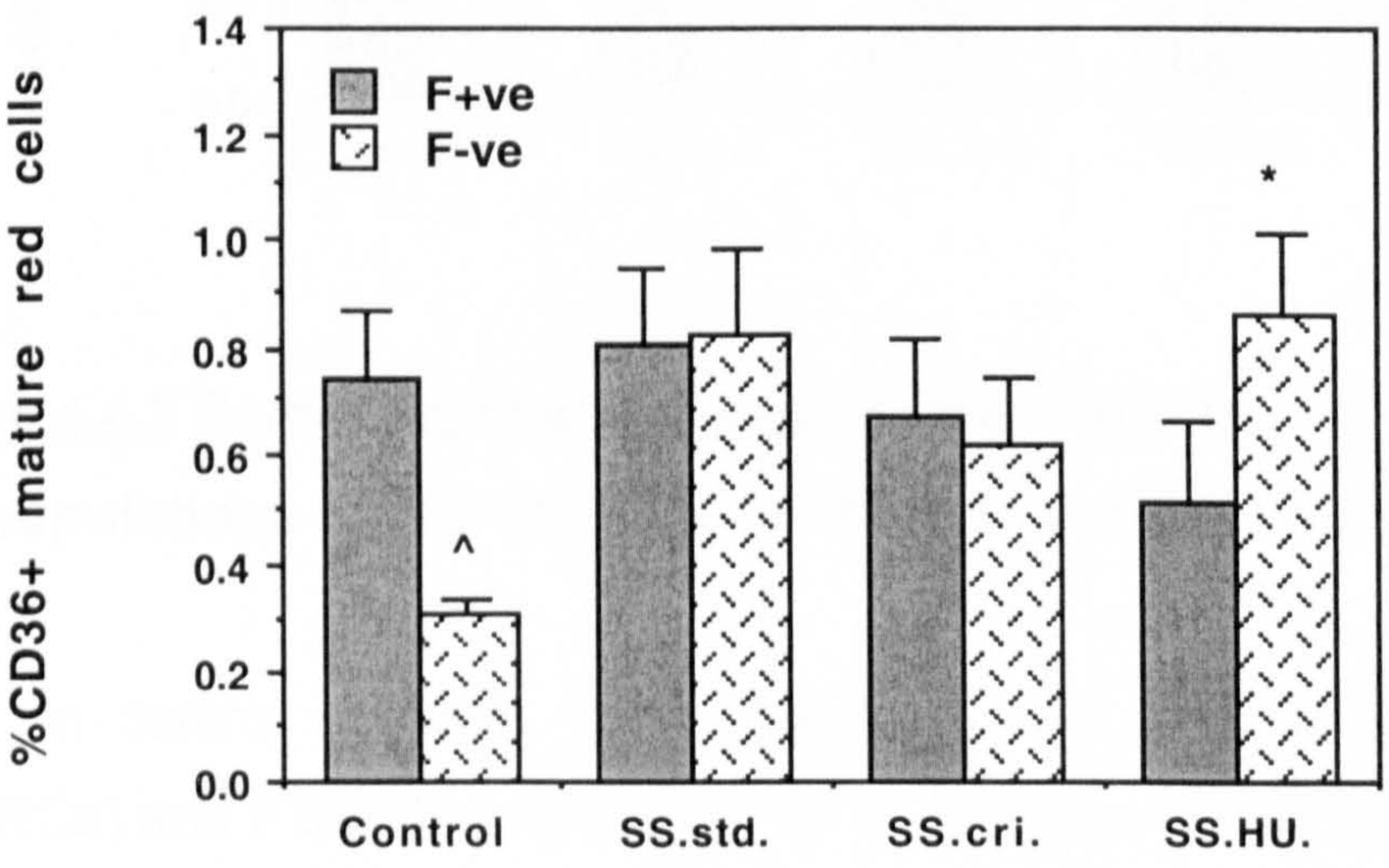
Having shown differences in AM expression in F⁺reticulocytes and F⁻ reticulocytes, analysis of this variable in mature red cells (R-) was undertaken to determine whether the same trend was observed in cells after the reticulocyte stage. (Table 4.4.2 and Figure 4.4.2)

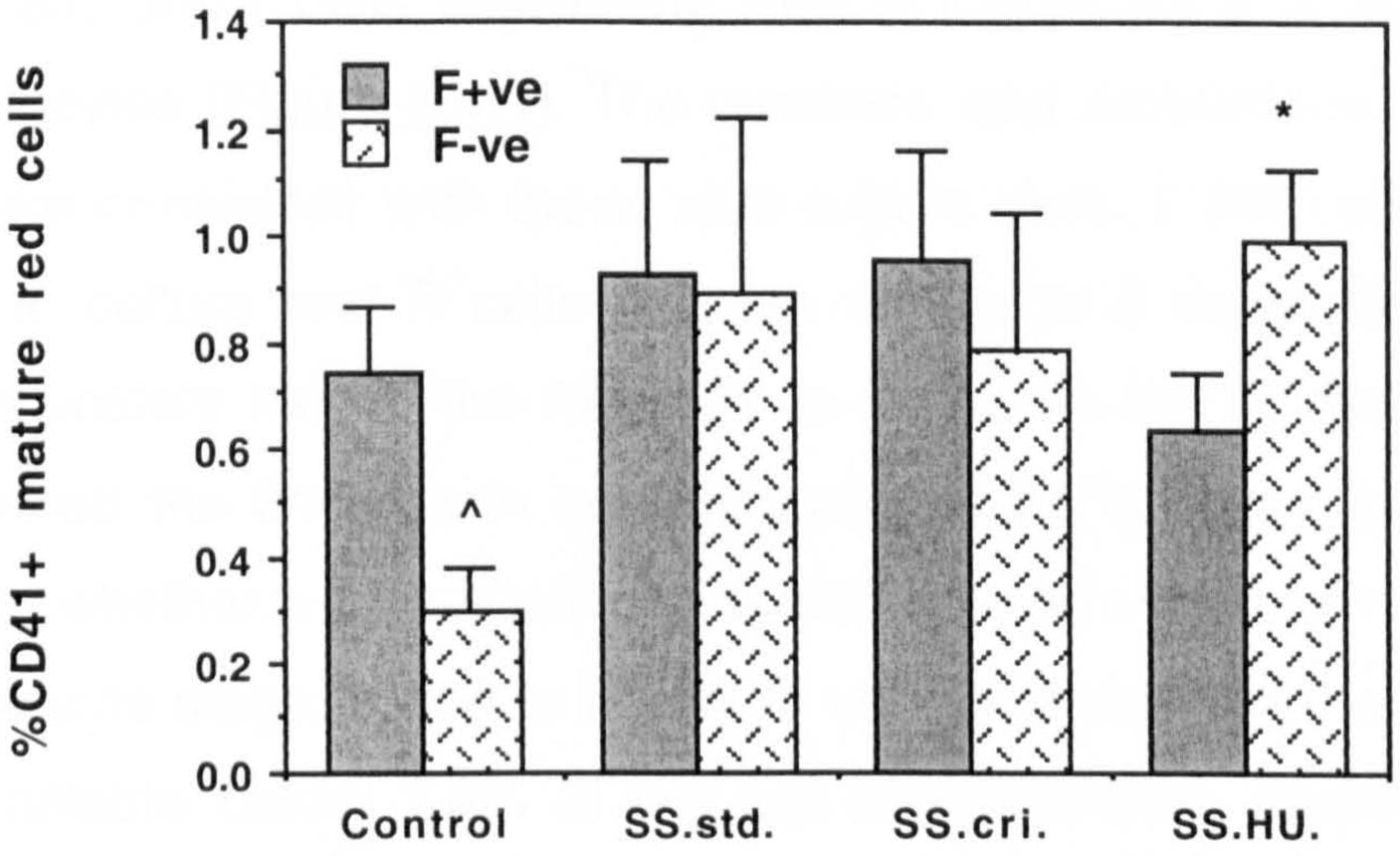
Table 4.4.2 Absolute numbers of triple variables in F^+mature red cell and F^- mature red cell populations

Parameter	Control (30)	SS.std.(35)	SS.cri. (22)	SS.HU.(10)
AM expressing F+ve mature red cells				
$CD36^+F^+$MRCs	1.47±0.15	6.20±0.71*	4.22±0.50*	8.03±1.75*
$CD41^+F^+$MRCs	1.46±0.16	7.21±1.05*	5.98±0.78*	9.84±1.02*
$CD49d^+F^+$MRCs	1.44±0.15	7.25±1.17*	5.01±0.64*	10.46±1.74*
AM expressing F-ve mature red cells				
$CD36^+F^-$MRCs	13.52±1.01	14.24±4.41	11.66±1.62	7.85±1.38#
$CD41^+F^-$MRCs	13.24±0.98	15.20±4.34	11.23±0.98	9.49±1.38#
$CD49d^+F^-$MRCs	13.81±1.06	15.16±4.05	13.56±2.12	9.70±1.39#
AM negative F+ve mature red cells				
$CD36^-F^+$MRCs	195.81±11.18	766.45±62.14*	621.27±51.35*	1568.05±112.47*§
$CD41^-F^+$MRCs	195.19±10.73	769.57±62.05*	618.00±52.37*	1528.09±094.70*§
$CD49d^-F^+$MRCs	192.73±10.19	771.15±62.68*	625.33±55.75*	1511.95±100.53*§
AM negative F-ve mature red cells				
$CD36^-F^-$MRCs	4419.03±69.07	1721.70±149.23^	1869.65±109.65^	910.93±113.99^#
$CD41^-F^-$MRCs	4416.50±69.19	1683.71±139.73^	1880.50±112.18^	943.58±125.58^#
$CD49d^-F^-$MRCs	4417.36±68.85	1761.79±143.43^	1862.76±113.88^	959.00±123.90^#

Absolute number at $x10^3$ cells/µl, SS. = Sickle cell patients, std. = in steady state, cri. = in crisis state, HU. = on hydroxyurea, F = HbF (foetal haemoglobin), MRCs = mature red cells or RNA^-cells

* significantly higher than control $p<0.05$, § significantly higher than SS.std. $p<0.05$, ^ significantly lower than control $p<0.01$, # significantly lower than SS.std. $p<0.05$,





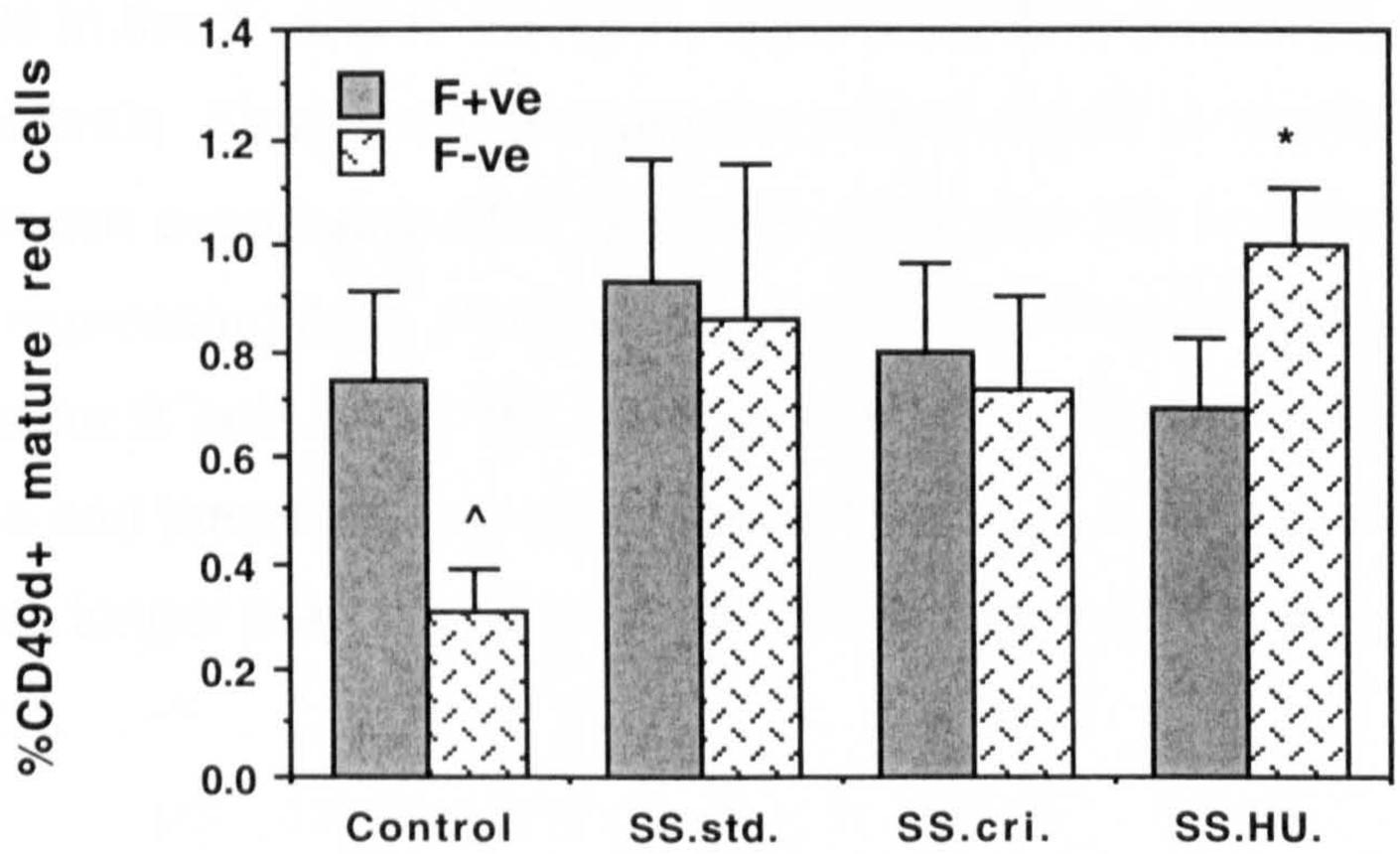


Figure 4.4.2 Percentages of AM expressing F^+mature red cell and F^-mature red cell populations CD36 (top), CD41 (middle) and CD49d (bottom).

In control subjects, the numbers of AM expressing F^+mature red cells (F^+MRCs) and F^-MRCs are similar as in reticulocyte population. This is possibly because R^-cells (MRCs) exceed R^+cells (reticulocytes) in control subjects by 50-100 fold, so the proportion of R^-cells expressing AMs in both F^+cell and F^-cell

populations should be about 0.1-0.2 times that of R^+cells. Indeed the proportion of F^+cells and F^-cells expressing AMs in Figure 4.4.2 is about 0.2 times that of reticulocytes (Figure 4.4.1). The numbers and proportions of R^-cells expressing AMs are consistent with the *in vitro* culture data. If AM^+cells is seen for up to 5 days in culture and R^+cells is seen for up to 3 days, then one would expect approximately half of the AM^+cells to reside in the R^+ and R^- fractions. This is confirmed the findings in control subjects in Tables 4.4.1 and 4.4.2. It is not certain whether a presentation of a small %AM^+cells beyond a few days after the reticulocyte stage is due to a lack of validity of detection by flow-cytometry, which is unreliable below 1.0% of any cell subpopulation. Nevertheless, the very low proportions of R^-cells expressing AMs after 5 days in culture is consistent both with the observed rate of AM loss in the controls and SS patients.

As with the reticulocyte (R^+) population, the trend of relative proportions of R^-cells in the F^+ and F^- fractions expressing AMs is similar in SS and HU-treated SS patients. Thus there are approximately equal proportions of F^+ and F^- in R^- population expressing AMs in steady state patients and lower % of F^+cells than F^- cells expressing AMs after HU treatment. The likely explanation for this is similar to that for R^+cells. After HU treatment, F^+MRCs have a survival advantage over F^- MRCs and therefore a smaller proportion of F^+cells still carry AMs, because they survive longer then they have more time to shed more AMs.

4.5. PAIRED DATA ANALYSIS IN STEADY STATE AND CRISIS PATIENTS

Rationale

In the previous sections, there was no significant difference in AM expression in F^+cells and F^-cells between SS in steady state or in crisis. However, in those previous sections, the comparison between sickle patients in steady state and in crisis was done in different groups of patients. Therefore values were not compared in and out of crisis in the same patients and it is possible that trends in AM expression could have been obscured by an unintentional selection bias between the two groups. In this section, the comparison was done on the same patients during their steady state and vaso-occlusive crisis.

Study design

A total of 7 pairs of sickle cell patient samples in their steady state (SS.std.) and crisis state (SS.cri.) were collected and stored at 4°C. The samples were assayed using triple-colour staining flow-cytometry (section 3.4.3) on the day of collection or not later than 3 days after collection. Paired data were compared between these two groups of samples.

Results and discussion

Comparison of paired data for the same sickle cell patients in steady state (SS.std.) and in crisis state (SS.cri.) are shown in Table 4.5. There is no significant difference between SS.std. and SS.cri. for any parameter in this group of patients. Thus in both paired and unpaired studies there is no difference in AM expression in any subpopulation of red cells (F+, F-, R+, R-). This suggests that vaso-occlusion does not result in a selective removal of populations expressing AMs or HbF. This does not exclude small numbers of cells being removal locally at vaso-occlusion sites but suggests that there is not a generalised process of removal of specific red cell populations on the basis of AM expression or HbF content during vaso-occlusion.

Table 4.5.1 Paired data of sickle cell patients in steady state and in crisis

	Parameter	SS.std. (7)	SS.cri.(7)
Single	**variables**		
	RBCs (10^6/μl)	2.71±0.12	2.64±0.21
	WBCs	12.73±1.38	14.09±1.78
	Plts	420.43±59.41	371.43±41.15
	MCV (fl)	85.30±2.17	84.07±3.02
	MCH (pg)	28.73±1.04	28.14±1.13
	Hb (g/dl)	7.73±0.35	7.13±0.43
	HbF (%)	4.17±0.92	4.67±1.00
	HbF/F$^+$cells (pg)	5.07±0.70	5.29±0.52
	%Retics	9.44±0.99	10.57±1.22
	%F$^+$cells	22.59±2.8429	22.80±3.83
	%CD36$^+$cells	2.77±0.19	3.30±0.56
	%CD41$^+$cells	2.72±0.17	2.64±0.39
	%CD49d$^+$cells	2.88±0.30	3.42±0.69
Double	**variables**		
	%F$^+$retics	9.21±0.88	10.96±2.35
AM expressing reticulocytes			
	%CD36$^+$retics	26.78±4.01	24.73±4.22
	%CD41$^+$retics	25.64±3.69	21.74±4.86
	%CD49d$^+$retics	26.50±4.69	24.13±3.85
AM expressing mature red cells			
	%CD36$^+$MRCs	0.55±0.07	0.88±0.14
	%CD41$^+$MRCs	0.59±0.05	0.70±0.15
	%CD49d$^+$MRCs	0.63±0.11	1.07±0.31
AM expressing F$^+$cells			
	%CD36$^+$(F$^+$cells)	1.41±0.41	2.37±0.62
	%CD41$^+$(F$^+$cells)	1.45±0.48	2.19±0.48
	%CD49d$^+$(F$^+$cells)	1.35±0.41	2.02±0.32
AM expressing F negative cells			
	%CD36$^+$(F$^-$cells)	3.20±0.32	3.73±0.73
	%CD41$^+$(F$^-$cells)	3.14±0.29	2.92±0.54
	%CD49d$^+$(F$^-$cells)	3.36±0.43	3.99±0.91

SS. = Sickle cell patients, std. = in steady state, cri. = in crisis state, AM = adhesion molecule, F = foetal haemoglobin (HbF), Retics = reticulocytes, MRCs = mature red cells (R^- or RNA^-)

4.6. PAIRED AND SEQUENTIAL DATA FOR PRE- AND POST-HYDROXYUREA TREATMENT

In the previous sections, the effect of hydroxyurea (HU) on AM expression in red cell sub-populations was compared for two groups of patients either on or off HU treatment but not done in the same patients. Therefore values were not exactly comparable and it is possible that trends in AM expression could have been obscured by an unintentional selection bias between the two groups. In this section, the comparison was done on the same patients before starting HU therapy and at the steady high levels of HbF after a period of HU therapy.

4.6.1. Paired data analysis in pre- and post-hydroxyurea treatment in the same patients

Study design

A total of 5 pairs of samples from 5 patients before HU therapy (SS.pre.) and after a period of HU therapy (SS.post) were collected. HU doses ranged from 0.5 to 1.5 g/day at mean duration of 34 weeks (range 30-40 weeks). The samples were tested using triple-colour staining flow cytometry (section 3.4.3) on the day of collection or not later than 3 days after collection. Paired data were compared between these two groups of blood samples.

Table 4.6.1.1 General data for hydroxyurea treated sickle cell patients

Patient	Age years	Sex	Diagnosis	Dose g/d	Duration weeks
1.	12	male	Sβ° thal	0.5	36
2.	57	male	SS	0.5-1.0	32
3.	22	female	SS	0.5-1.0	40
4.	22	female	Sβ° thal	0.5-1.0	30
5.	52	female	SS	1.5	32

Results and discussion

Comparison of paired data for the same sickle cell patients before (SS.pre.) and after (SS.post.) HU therapy are shown in Tables 4.6.1.2 and 4.6.1.3. In Table 4.6.1.2, a large number of statistically significant differences are seen before and after HU treatment, which are largely in agreement with reports of the effect of HU in SS patients previously reported (Charache *et al.*, 1992; Shetty *et al.*, 1998; Steinberg *et al.*, 1997) as well as being in agreement with the unpaired data in section 4.3. Thus there is a significant reduction in white blood cell count (WBC), platelet count (Plts) and reticulocyte count (retics) after HU treatment. There is also a significant increase in MCV, HbF and F^+cells post HU treatment. Conversely, there is a significant reduction in the % and absolute numbers of total red cells expressing CD36, CD41 or CD49d but only absolute numbers of reticulocytes expressing AMs are significantly reduced. A reductions in $\%CD36^+$reticulocytes with increment in $\%CD36^+$mature red cells as well as reductions in $\%CD49d^+$mature red cells and *$\%CD49d^+$reticulocytes after HU* treatment have been reported previously (Styles *et al.*, 1997). However in that report, CD41 has not been investigated following HU treatment.

Analysis shows that average HbF content per F^+cell (F/Fc) doubles post HU (from 7.6 to 13.1 pg/cells), similar to the finding in a previous report (Maier-Redelsperger *et al.*, 1998). This suggests that not only do the number of F^+cells increase but the content of HbF per F^+cell also increases. These findings suggest that prior to HU treatment about one quarter of the Hb in a F^+cell is HbF on average, whereas post HU treatment nearly half the Hb in an F^+cell is HbF. This increased HbF content would be expected to slow the rate of HbS polymerisation in each F^+cell. The $\%F^+$reticulocytes increases post HU treatment, in agreement with previous reports (Dover *et al.*, 1986; Osterhout *et al.*, 1996) but the absolute number of F^+reticulocytes (F^+R^+) has decreased, because the number of total reticulocytes has decreased (from 376 to 172 $x10^3$ cells/cu.mm.).

Table 4.6.1.2 Paired data for pre- and post-hydroxyurea treatment

Parameter	SS.pre. (5)		SS.post.(5)	
	Percent (%)	Number ($x10^3/\mu l$)	Percent (%)	Number ($x10^3/\mu l$)
Single variables				
RBCs	NA	3060.00±494.00	NA	2806.00±400.00
WBCs	NA	10.42±2.12	NA	8.18±1.35^
Plts	NA	484.20±158.40	NA	379.20±130.74^
MCV	NA	85.06±8.02	NA	96.12±5.86*
MCH (pg)	NA	27.92±3.45	NA	31.98±2.37*
Hb (g/dl)	NA	8.02±0.68	NA	8.64±0.57
HbF (g/dl)	11.68±1.61	0.94±0.15	26.78±2.20*	2.35±0.32*
HbF/Fc (pg)	NA	7.56±0.60	NA	13.09±1.20*
F^+cells	42.70±6.30	1251.52±248.42	65.50±4.57*	1800.65±202.12*
Retics	13.11±2.98	376.33±79.27	6.30±1.35^	172.86±35.84^
$CD36^+$cells	5.55±1.59	186.31±71.81	2.54±0.34^	72.45±14.74^
$CD41^+$cells	5.57±1.60	186.43±70.52	2.45±0.43^	69.96±15.60^
$CD49d^+$cells	5.83±1.60	195.87±72.02	2.68±0.39^	76.33±16.07^
Double variables				
F^+retics (R^+)	21.67±3.56	73.75±13.81	33.36±4.70*	57.96±8.26
F^+MRCs (R^-)	46.36±7.61	1177.76±244.93	67.86±4.68*	1748.69±198.50*
$R^+(F^+$cells)	6.51±1.39	73.75±13.81	2.96±0.48	51.96±8.26
$R^+(F^-$cells)	19.53±6.11	296.69±70.36	12.92±3.55	120.90±31.00
AM expressing reticulocytes				
$CD36^+$retics (R^+)	31.55±6.98	110.00±35.74	26.78±3.85	45.56±10.32^
$CD41^+$retics (R^+)	27.75±5.56	106.71±34.22	26.41±3.79	43.35±10.41^
$CD49d^+$retics (R^+)	30.58±6.35	113.11±36.58	26.77±4.40	44.90±11.21^
AM expressing mature red cells				
$CD36^+$MRCs (R^-)	2.38±0.99	76.35±35.89	1.02±0.14^	26.89±4.79^
$CD41^+$MRCs (R^-)	2.57±1.08	79.72±37.10	1.01±0.19^	26.61±5.58^
$CD49d^+$MRCs (R^-)	2.73±1.11	82.75±36.44	1.19±0.13^	31.42±5.27^
AM expressing F^+cells				
$CD36^+$ (F^+cells)	2.91±0.73	36.98±13.02	1.43±0.34^	25.84±6.14
$CD41^+$ (F^+cells)	3.19±0.93	40.10±13.02	1.45±0.39^	25.32±5.43
$CD49d^+$ (F^+cells)	3.90±1.35	45.37±15.02	1.56±0.28^	28.15±5.22
AM expressing F negative cells				
$CD36^+$ (F^-cells)	7.25±1.73	149.37±64.00	4.69±0.42^	46.62±10.08^
$CD41^+$ (F^-cells)	7.21±1.65	146.34±61.46	4.82±0.47^	44.64±11.37^
$CD49d^+$ (F^-cells)	7.26±1.48	150.49±60.21	4.84±0.56^	48.17±11.92^

All absolute cell numbers = $x10^3$ cells / µl of blood, SS. = sickle cell patients, pre. = pre-hydroxyurea, post. = post-hydroxyurea, RBCs = red cell count, WBCs = white cell count, Plts = platelet count, Hb = haemoglobin, HbF = foetal haemoglobin, HbF/Fc = HbF per F^+cell, F = foetal haemoglobin (HbF), Retics = reticulocytes, R = RNA (reticulocytes), MRCs = mature red cells.
^ significantly lower than pre-HU treatment, * significantly higher than pre-HU treatment

Table 4.6.1.3 Paired data of triple variables in absolute numbers for pre- and post-hydroxyurea treatment

Parameter	SS.pre. (5)	SS.post.(5)
AM expressing F+ve reticulocytes		
$CD36^+F^+$Retics	14.01±2.75	9.75±3.04
$CD41^+F^+$Retics	14.30±2.03	9.44±3.09
$CD49d^+F^+$Retics	15.50±3.62	9.70±2.60
AM negative F+ve reticulocytes		
$CD36^-F^+$Retics	60.90±14.91	42.42±7.40
$CD41^-F^+$Retics	61.77±11.96	42.39±5.76
$CD49d^-F^+$Retics	57.49±13.45	41.66±6.22
AM expressing F-ve reticulocytes		
$CD36^+F^-$Retics	95.92±34.78	35.82±8.23^
$CD41^+F^-$Retics	92.41±32.62	33.92±8.65^
$CD49d^+F^-$Retics	97.62±33.41	34.68±9.37^
AM negative F-ve reticulocytes		
$CD36^-F^-$Retics	187.64±44.80	88.03±23.10^
$CD41^-F^-$Retics	224.27±56.11	86.65±29.80^
$CD49d^-F^-$Retics	207.11±49.82	83.59±22.44^
AM expressing F+ve mature red cells		
$CD36^+F^+$MRCs	23.05±12.28	16.09±3.18
$CD41^+F^+$MRCs	25.79±12.10	15.88±2.80
$CD49d^+F^+$MRCs	29.89±13.30	17.94±2.79
AM negative F+ve mature red cells		
$CD36^-F^+$MRCs	1169.41±254.50	1736.82±210.08*
$CD41^-F^+$MRCs	1175.58±245.05	1767.07±193.88*
$CD49d^-F^+$MRCs	1107.00±204.88	1746.05±185.61*
AM expressing F-ve mature red cells		
$CD36^+F^-$MRCs	53.37±29.33	10.80±2.32^
$CD41^+F^-$MRCs	53.93±28.87	10.72±3.45^
$CD49d^+F^-$MRCs	52.89±26.82	13.49±3.34^
AM negative F-ve mature red cells		
$CD36^-F^-$MRCs	1455.70±313.93	866.27±210.77^
$CD41^-F^-$MRCs	1411.95±289.19	839.93±260.97^
$CD49d^-F^-$MRCs	1492.54±330.25	859.89±233.83^

All absolute cell numbers are shown in $x10^3$ cells / µl of blood, SS. = sickle cell patients, pre. = pre-hydroxyurea, post. = post-hydroxyurea, Hb = haemoglobin, HbF = foetal haemoglobin, HbF/Fc = HbF per F^+cell, F = foetal haemoglobin (HbF), Retics = reticulocytes, R = RNA (reticulocytes), MRCs = mature red cells
^ significantly lower than pre-HU treatment, * significantly higher than pre-HU treatment

The mean RNA fluorescent intensity in various red cell sub-populations was determined for these 5 paired samples of pre- and post-hydroxyurea treatment, in order to estimate age different among these sub-populations. (Table 4.6.1.4)

Table 4.6.1.4 Mean RNA fluorescent intensity in red cell sub-populations of pre- and post hydroxyurea treatment

Sub-population		F^+	F^-	$F^+ + F^-$
Retics (R^+)	Pre-HU	2.358±0.243	2.772±0.234	2.652±0.259
	Post-HU	2.416±0.187	3.692±0.320*	3.224±0.213
MRCS (R^-)	Pre-HU	0.133±0.009	0.146±0.013	0.138±0.013
	Post-HU	0.161±0.014	0.178±0.019	0.166±0.015
All red cells	Pre-HU	0.280±0.037	0.602±0.172*	0.441±0.088
	Post-HU	0.233±0.022	0.579±0.133*	0.344±0.063^

* Significantly higher than F^+ population ($p<0.05$),
^ Significantly lower than pre-HU ($p<0.05$)
Retics = reticulocytes, MRCs = mature red cells

In mature red cells, the number and proportion of cells expressing HbF increases significantly. As previously discussed, AM expression in reticulocytes decreases significantly in terms of absolute numbers but not in % values. This is partly in agreement with a previous report (Styles *et al.*, 1997), who found reduction in both number and % of reticulocyte expressing CD49d after HU treatment. In this thesis the degree of change for % CD36 and CD49d expressing reticulocytes is less than that report.

Changes in AM expression in F^+cell and F^-cell populations have not been previously compared. In Table 4.6.1.2, there are significant decreases in % of both F^+cell and F^-cell populations expressing AMs post HU treatment, comparable with the unpaired data (Tables 4.2.4 and 4.3.3.4 for data obtained from double- and triple-colour staining procedures respectively). The %AM expressing F^+cells has decreased more than the %AM expressing F^-cells. This

was likely to be secondary to the survival advantage of F^+cells with higher HbF content leading to an older age distribution and therefore greater AM loss after HU treatment. In other words F^-cells are younger than F^+cells. This hypothesis is confirmed by a significant higher in the mean RNA fluorescent intensity (reflecting more RNA content) in F^-cells than F^+cells (Table. 4.6.1.4). It is also confirmed again by the higher %reticulocytes (R^+) in F^-cells than the F^+cells in both pre- and post-HU treatment (Table 4.6.1.2). The findings in this paired study are consistent with this hypothesis. However the absolute numbers of AM expressing F^+cells are not significantly reduced after HU treatment, probably because the total number of F^+cells is increased. Unlike the significant reduction in the absolute numbers of AM expressing F^-cells, because the total number of F^-cells is also reduce after HU treatment.

Table 4.6.1.3 shows the data obtained from 3 colour analysis in absolute values, displayed in a similar layout to the results obtained using unpaired patient groups in Tables 4.4.1 and 4.4.2. The same trends are largely present in the paired samples as in the unpaired samples. The most significant reductions in absolute numbers are found in the F^-cell populations, both R^+ (reticulocyte) and R^- (MRCs) as well as AM^+ and AM^-. These reductions in F^+cell population are generally less marked, possibly because total F^+cells are increased. However, the post HU increments for AM^-F^+mature red cells are clearly marked. This subpopulation is the one predicted to be of most benefit to the patients because of the high HbF content and the absence of AMs.

The percentages of reticulocytes expressing AMs in the F^+cell and F^-cell populations of pre- and post-HU treated patients are shown in Figure 4.6.1.1. The $\%AM^+$reticulocytes seem to decrease in both F^+reticulocytes and F^- reticulocytes post-HU treatment but does not reach the significant level. However, the trend of reduction is higher in F^+reticulocytes than F^-reticulocytes consistent with the finding that F^+reticulocytes are older because they survive longer and therefore they lose more AMs. The mean RNA fluorescent intensity for F^+reticulocytes is shown significantly lower than F^-reticulocytes post-HU treatment, which is consistent with this hypothesis (Table 4.6.1.4).

The %mature red cells expressing AMs in the F^+cell and F^-cell populations, pre- and post-HU treatment are shown in Figure 4.6.1.2. AM expression in F^+cells falls but not in F^-cells. This is again compatible with a survival advantage of F^+cells leading to an older population with less AM expression or more AM shedding. By contrast AM expression in F^-cells has not changed after HU treatment. This suggests that the conclusion of Styles et al. 1997 that changes in AM expression post-HU treatment are due to a decrease in %AM^+reticulocyte populations is unlikely, because these cells have no survival advantage in sickle cell patients. We conclude that the main reduction in AM expression post HU is secondary to a decrease in total reticulocytes and an increase in F^+cells and HbF content in F^+cells, leading to better survival of both reticulocytes and red cells after the reticulocyte stage.

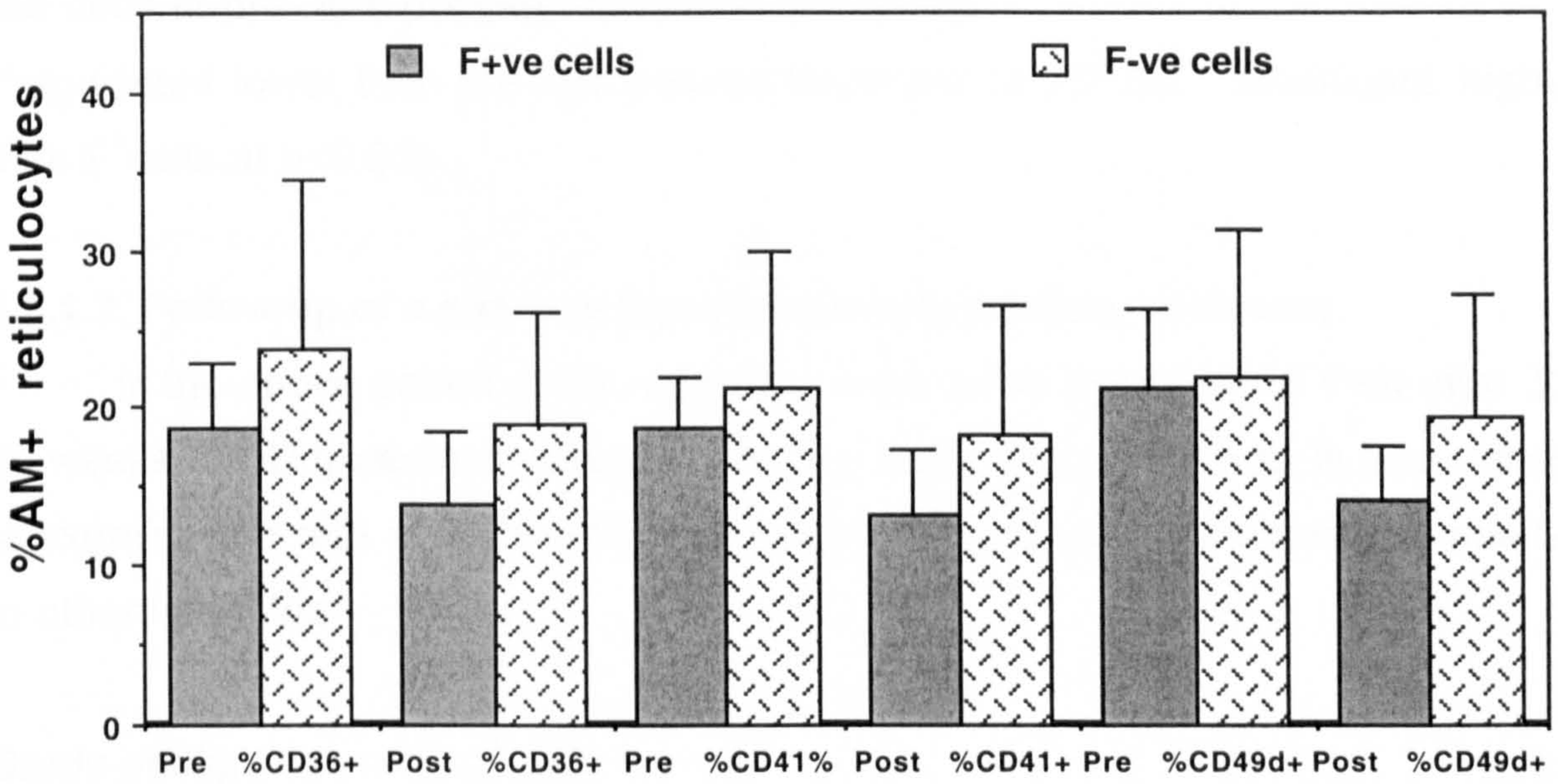


Figure 4.6.1.1 Percentages of AM expressing F^+reticulocytes and F^- reticulocytes in pre- and post-hydroxyurea treatment

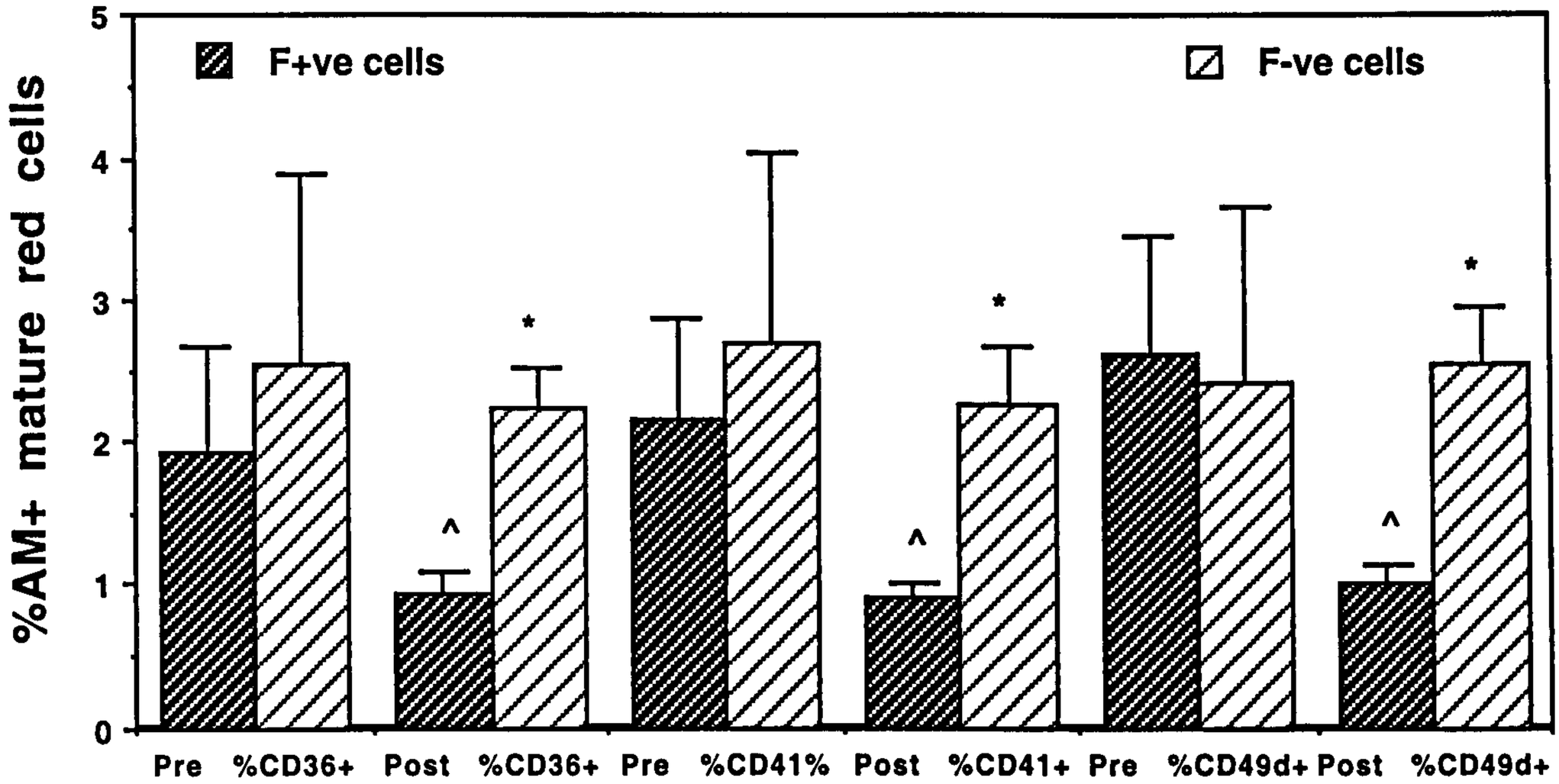


Figure 4.6.1.2 Percentages of AM expressing F^+mature red cells and F^-mature red cells in pre- and post-hydroxyurea treatment

(^significant lower than pre-hydroxyurea treatment at $p<0.05$; *significant higher than F^+cells at $p<0.05$)

4.6.1.2. Follow up of a sickle cell patient on hydroxyurea treatment

In the above paired study, samples were taken pre-HU and then after 30-40 weeks of HU treatment. A supplementary study was performed in one patient to examine the rate at which AM expression changed with HU treatment, relative to other variables.

Study design

A 12 year old male with $S\beta^o$ thal of Cypriot origin was followed before HU was given (defined as day 0) and on days 14, 28, 49, 84, 97, 140, 161 and 273 of HU therapy. Oral HU was given at 500 mg/day from day 0 to Day 273. All blood samples were assayed using triple-colour flow cytometry (section 3.4.3) within 3 days after collection. This is the first patient in Table 4.6.1.

Results and discussion

The %AM expressing cells fell at approximately the same rates in reticulocytes and mature red cells. Also the rate of decrease in AM expressing cells appears similar in both F^+cell and F^-cell populations. In all of these subpopulations, the nadir value for %AM expression is achieved at about 140 days (20 weeks or 5 months) of treatment. Styles et al. 1997, found that nadir values of CD49d were achieved at 3 months on HU (15mg/k/day) therapy.

It is interesting that HbF and F^+cells continue to rise after the nadir point of AM expression and is still increasing at 273 days (39 weeks or nearly 10 months). By contrast, the nadir reticulocyte count is reached sooner at about 84 days (12 weeks or 3 months) after starting the HU therapy, while MCV and %HbF were still rising. In Styles's report, AM expression was not compared in F^+cell and F^-cell populations.

There are a number of kinetic differences between this thesis and Styles's report. While the kinetics of changes in AM expression on reticulocytes in this thesis are similar to those for CD49d in Styles's report, but values for CD36 in all populations are higher in Styles's report. However, an increase in $CD36^+$mature red cells in Styles's report is disagreement with the finding in this thesis where they were significantly reduced after HU treatment (Table 4.6.2.2). Since the kinetics and % changes were similar for all 3 AMs in this thesis. Therefore the values show in this thesis are possibly more valid. Antibodies to AMs used in Styles's report are different from those used this thesis, which could explain the differences in % cells stained. The findings in this thesis with 3 independent (rather than 2) AMs shows almost exactly the same changes with all 3 AMs suggesting a similar mechanism of regulation.

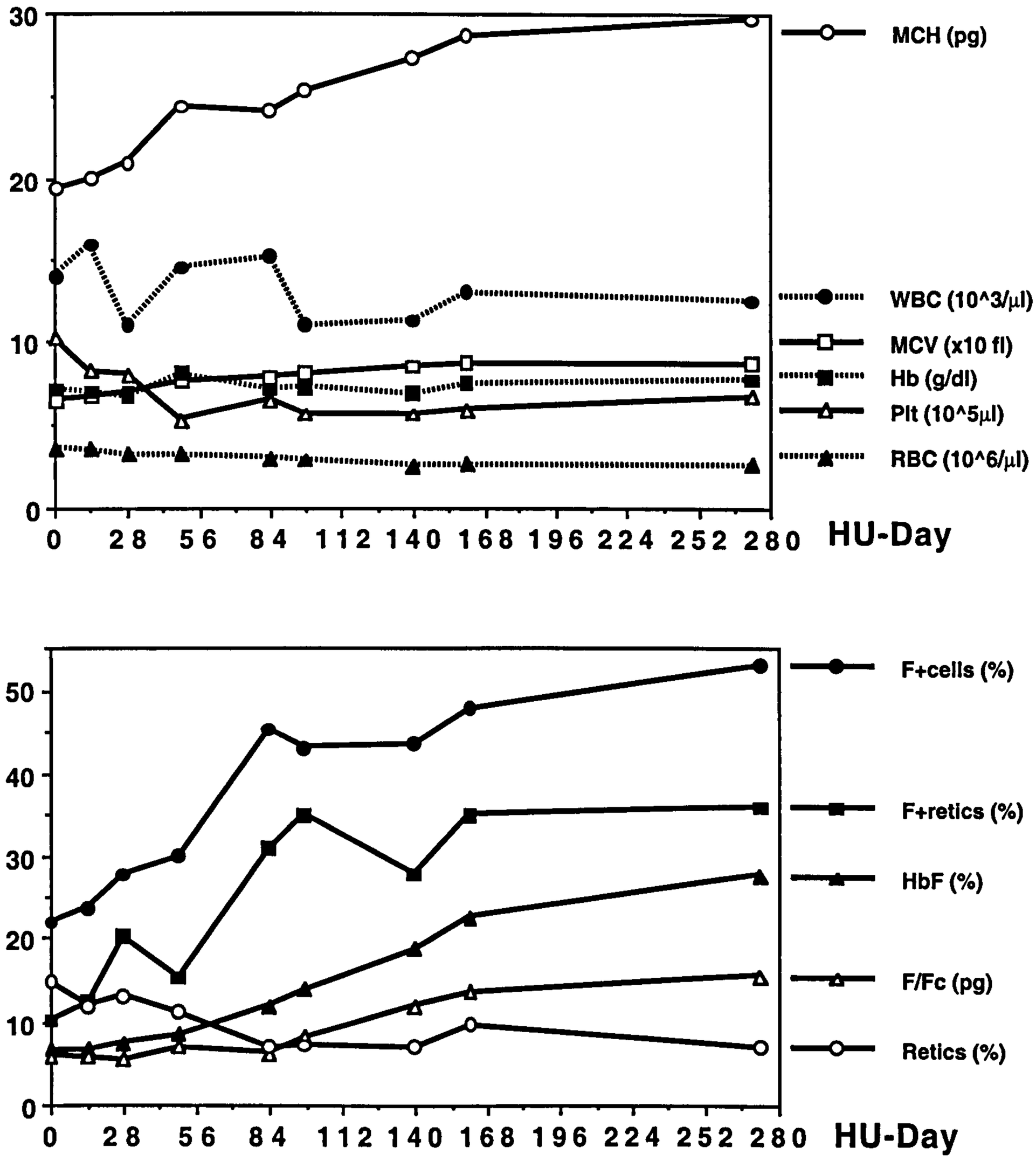


Figure 4.6.2.1 Follow up data of a sickle cell patient during Hydroxyurea treatment (cells = red blood cells, retics = reticulocytes, F/Fc = HbF per F^+cells)

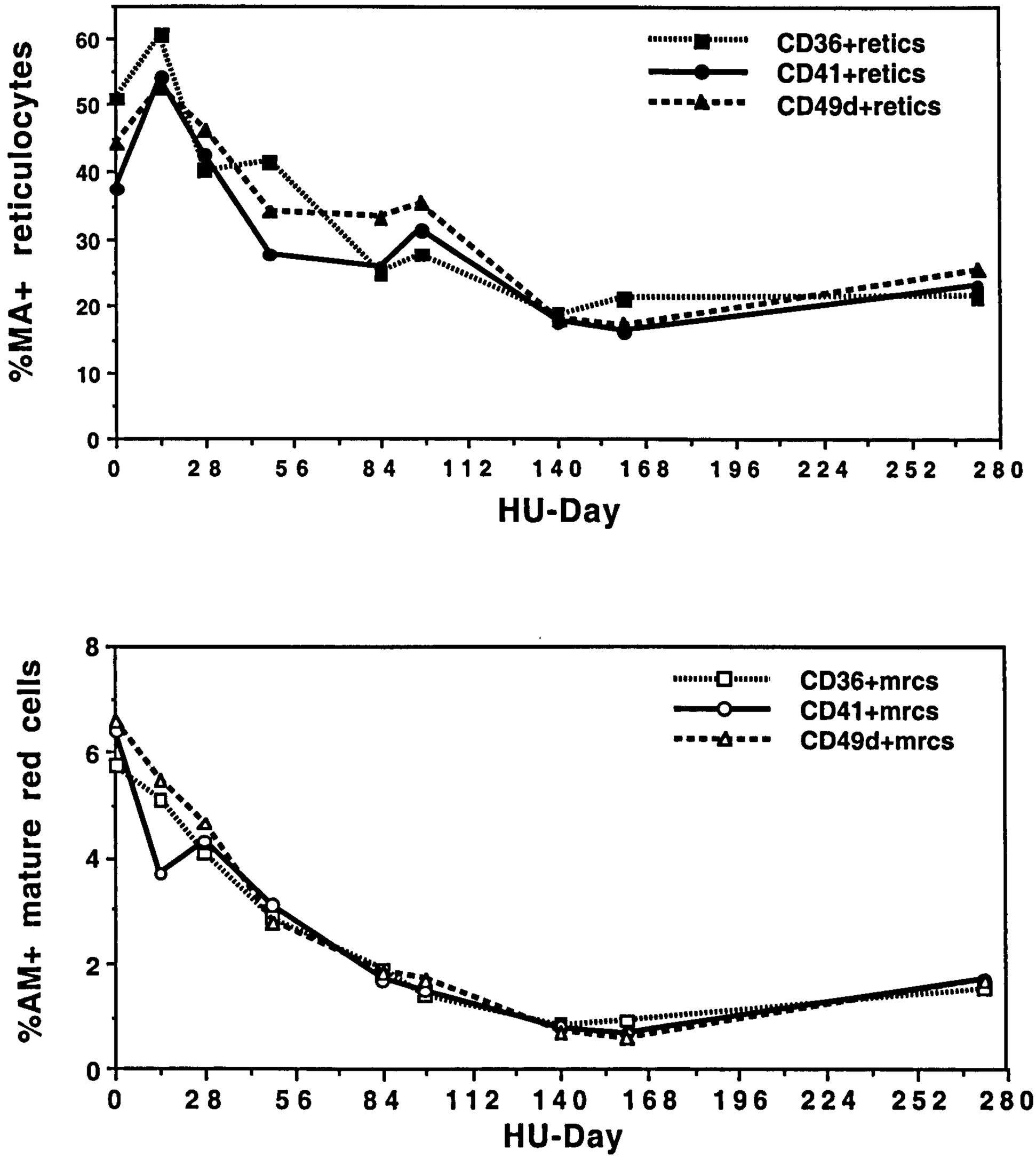


Figure 4.6.2.2 Follow up percentages of AM expressing reticulocytes (top) and mature red cells (bottom) (Retics = reticulocytes, mrcs = mature red cells)

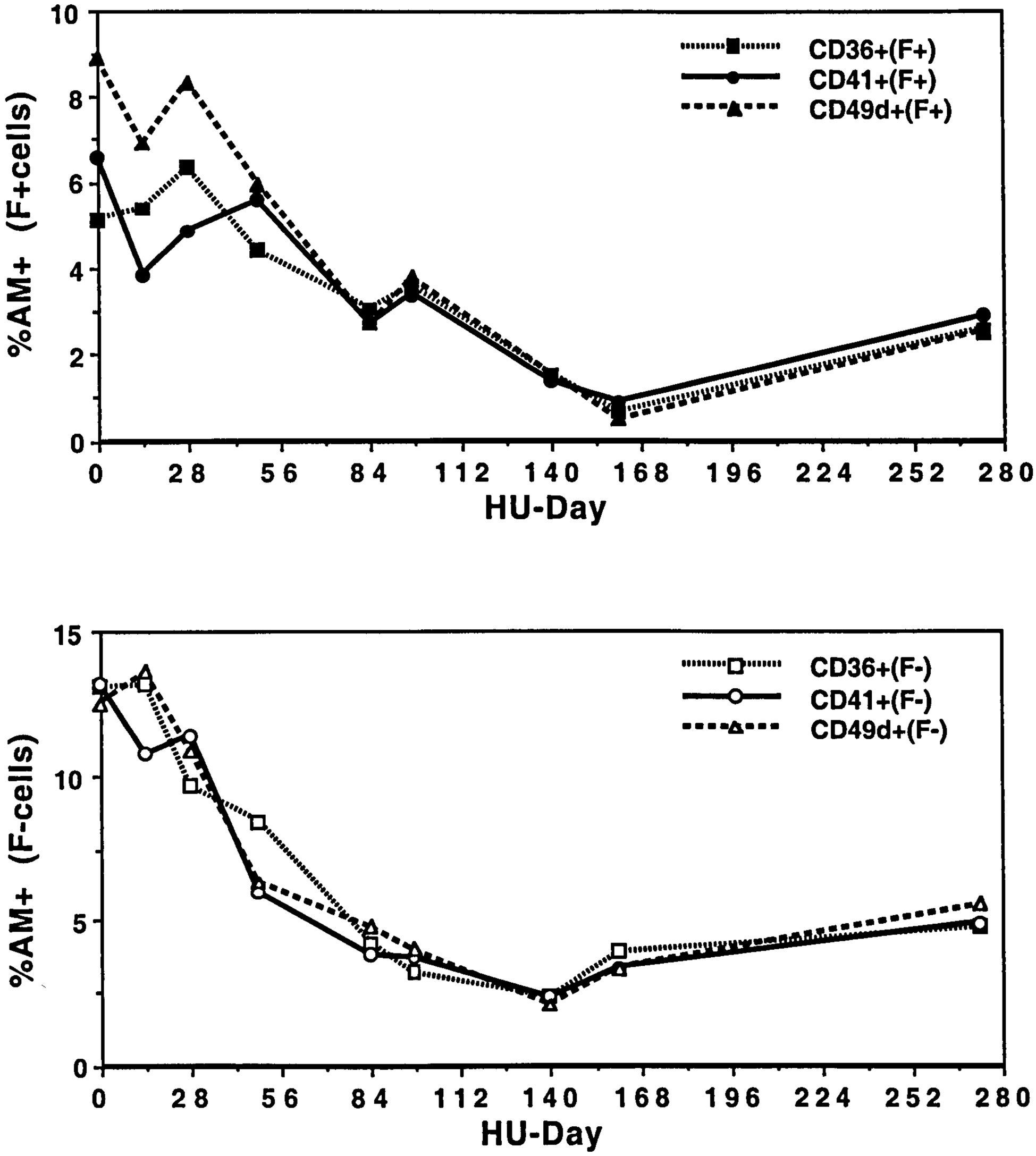


Figure 4.6.2.3 Follow up percentages of AM expressing F^+cells (top) and F^-cells (bottom)

Styles concluded that changes in adhesion molecule expression were not caused by a decrease in reticulocytosis with HU therapy. This was based on the observations that the % of CD36 and CD49d^{+}retciculocytes appeared to decrease post-HU treatment but that mean cell RNA fluorescence did not change with HU therapy. In this thesis however, the % of reticulocytes expressing AMs pre- and post-HU treatment are not significantly different either in the non-paired analysis (Figure 4.2.2) or in the paired analysis (Table 4.3.3.3). Our findings show that the mean reduction in the % and absolute number of reticulocytes at 30-40 weeks is 52% and 54% respectively. The reduction in %reticulocytes expressing AMs was only 15% whereas the decrease in absolute number was 61%.

In F^{+}cells however, there is a significant reduction in the proportion of cells expressing AMs (Figure 4.2.4, Tables 4.2.4 and 4.6.1.2). Although in this thesis a lack of change in mean reticulocyte RNA fluorescence pre- and post-HU treatment (Table 4.6.1.4) was also found. However, the influence of HbF content as a separate variable has been studied.

In Table 4.6.3, mean cell fluorescence is significantly higher in F^{-}cells than F^{+}cells (both in reticulocyte stage and mature red cell stage), consistent with an older age and survival advantage of F^{+}cells. It is also clear that the proportion of F^{+}cells and F^{+}reticulocytes increases post-HU (Table 4.6.1.2). Thus the decrease in AM expression appears secondary to an increase in the proportion of F^{+}cells. This is consistent with the lower mean cell fluorescence in (combination of F^{+} and F^{-}) cells post-HU treatment of all cells (Table 4.6.1.4), reflecting a higher proportion of F^{+}cells. This argues against the conclusion of Styles *et al.* 1997 that effects on AM expression were 'independent of effects on reticulocyte age'. The findings in this thesis suggest that changes in AM expression in reticulocytes post-HU treatment are secondary to reduction of %reticulocytes and increments in F^{+}cells and HbF content in F^{+}cells, leading to a higher proportion of cells with a survival advantage and therefore lower AM expression.

4.7. CONCLUSIONS

In this chapter, the effect of HbF content as an independent variable on AM expression and cell survival beyond the reticulocyte stage have been observed in control subjects and in sickle cell patients before and after HU therapy. This has not been previously described and shows important differences in AM expression between F^+ and F^- populations.

It is clear that HbF content enriches for cells maturing beyond the reticulocyte stage in sickle patients but not in control subjects. This is the first use of flow cytometry for this purpose and the findings are consistent with other investigators about to the enrichment ratio (ER) (Dover *et al.*, 1978a; Dover *et al.*, 1978b). This ratio has not been described previously for patients on HU treatment. It appears that there is no additional increase in the enrichment ratio for patients on HU therapy.

The effect of red cell ageing on AM expression has not been studied previously in detail. In this thesis, previous findings that AM expression on mature red cells is less than on reticulocytes (Styles *et al.*, 1997) is confirmed. However *in vitro* culture shows that AMs are shed by reticulocytes at a rate slightly slower than the rate of RNA loss. This has not been described previously and suggests that AM expression is an indirect indicator of red cell age. Thus the higher the proportion of reticulocyte and red cells less than 1 week older than the reticulocyte stage, the higher the % of cells expressing AMs.

In this thesis 3 AMs have been measured independently unlike previous studies where only 2 AMs were measured (Styles *et al.*, 1997). The findings in this thesis indicate that all three AMs measured are expressed and are shed at similar frequencies and rates. The suggestions by Styles that different AMs respond to HU differently is not supported by the findings in this chapter.

Previously the influence of HbF content on AM expression has not been studied. This potentially important for two reasons. Firstly it is important to know whether F^+cells are qualitatively different in control subjects from F^-cells. In this thesis, it is clear that in control subjects AMs are expressed in a higher

proportion in F^+cells than in F^-cells (Figure 4.2.4) and in F^+reticulocytes than in F^- reticulocytes (Figure 4.4.1). This the first demonstration of a difference between F^+cells and F^-cells even though other differences have not been found. The finding (in healthy control subjects) is consistent with F^+cells being a population of cells which are less mature possibly having undergone less divisions prior to release from the bone marrow than F^-cells.

The second reason why HbF content being potentially important for understanding AM expression came from the studies in pre- and post-HU treatment. The findings in this chapter show that in sickle cell patients, unlike in control subjects, the %AM expressing cells is lower in F^+cell than F^-cell sub-populations. The likely explanation of this finding is the improved survival beyond the reticulocyte stage for F^+cells, which allows more AMs to shed. This observation is critical to the interpretation of the effects of HU treatment on AM expression on red cells. The findings in this chapter suggest that the reduced AM expression on red cells following HU treatment results from two independent effects. The first is a reduction in reticulocyte count. The second is secondary to increased proportions of F^+cells, which survive longer and therefore express less AMs.

CHAPTER 5

ENDOTHELIAL FACTORS IN SICKLE CELL DISORDERS

5.1. BACKGROUND

5.1.1. Endothelial factors and hypoxia in vaso-occlusion

In this chapter, the central hypothesis examined is whether local hypoxia resulting from vaso-occlusion increases vascular endothelial growth factor (VEGF) release from the microvasculature and adjacent ischaemic tissues, which in turn can be measured in the blood. A secondary hypothesis is whether hypoxia induced VEGF leads to a secondary increase in nitric oxide (NO) concentration. Hypoxia causes both the mRNA and VEGF protein increases in *ex vivo* perfusion lung tissues (Tuder *et al.*, 1995) and *in vitro* cultured hepatocytes (Sandner *et al.*, 1997). Hypoxia also increases the expression of a number of genes encoding vascular cell mitogens produced by vascular endothelial cells (VECs) including platelet-derived growth factor B (PDGF-B), endothelin-1 (ET-1), and VEGF (Kourembanas *et al.*, 1997). VEGF then upregulates endothelial nitric oxide synthase (eNOS) and stimulates NO production (Hood *et al.*, 1998). In human umbilical vein endothelial cells (HUVECs), the activation of VEGF receptor-2 (Flk-1/KDR) upregulates both eNOS and iNOS (inducible NOS), while stimulation of VEGF receptor-1 (Flt-1) did not generate such a signal. This indicates that VEGF can upregulate NO production and release from VECs and other cell types. If this hypothesis is correct, and other sources of VEGF and NO independent to local ischaemia do not swamp such changes, then the onset of vaso-occlusive crisis might be associated with an increase in both VEGF and NO levels in the blood. NO and VEGF are synthesised in a number of cell types, including endothelium and platelets. Both of these cells have been reported to be activated in sickle cell disorders. It is therefore also important to examine whether NO and VEGF levels are associated with changes in markers of endothelial and platelet activation.

5.2. LEVELS OF VEGF AND NOx IN SERUM AND PLASMA

5.2.1. Rationale

It was predicted that in sickle cell patients, localised vaso-occlusion could increase VEGF and NOx levels in the circulation. Vaso-occlusion and consequent local hypoxia could potentially induce VEGF production from a variety of cell types in the microcirculation, as VEGF synthesis is regulated by a hypoxia sensitive element.

Vaso-occlusion could also increase NO synthesis, which could be mediated in a variety of ways. In endothelial cells, hypoxia is known to induce eNOS, which is mediated by intracellular Ca^{2+} fluxes. Increased NO synthesis could then lead to compensatory vasodilatation. However eNOS can produce only small amounts of NO which may not be detectable as a change in the plasma level. Furthermore, NO is highly reactive with certain molecules that it encounters, most NO will interact with red cell haemoglobin to form nitrosyl-Hb, while a proportion will be rapidly metabolised to nitrites and nitrates, measured collectively as NOx. In non-endothelial cells, such as macrophages/monocytes, iNOS is activated by TNF-α, which has been reported to be increased in sickle cell patients (Malave, 1993; Francis, 1992). This could also increase plasma NOx levels.

In order to measure plasma VEGF and NOx, it was important to establish the optimal method for sample collection and whether values obtained were affected by the anticoagulant used. The levels of VEGF and NOx were therefore compared in serum, EDTA and CTAD plasma between healthy control subjects and sickle cell patients in steady state and in crisis.

5.2.2. VEGF levels in blood samples from different anticoagulants

Study design

Serum samples were collected from 16 healthy control subjects (control.), 10 sickle cell patients in steady state (SS.std.), 4 sickle cell patients in crisis (SS.cri.) and 4 sickle cell patients undergoing hydroxyurea treatment (SS.HU.). Steady state was defined as samples taken in outpatients who were pain free. Crisis state was defined as an acute painful episode requiring admission to hospital for management of pain. Hydroxyurea treatment was defined as receiving 0.5-2.0 g/day for several week and HbF levels reach a steady levels. All patients had samples taken for Hb typing and quantitation (by HPLC) and samples containing transfused red cells, as indicated by the presence of HbA, were excluded. The EDTA plasma samples were collected from 20 control, 8 SS.std., 8 SS.cri. and 7 SS.HU. The CTAD plasma samples were collected from 21 control., 13 SS.std., 7 SS.cri. and 8 SS.HU. Control blood samples were taken from University College London postgraduate students and laboratory personnel. Sickle blood samples were taken from patients at University College London Hospitals (UCLH). Serum, EDTA and CTAD plasma were stored at -70°C. Full blood count (FBC) with reticulocyte count was assayed on a blood cell analyser (Sysmex SE-9500). Samples were assayed for VEGF and NOx levels as described in Chapter 2.

Results

Levels of VEGF (pg/ml) in normal controls, SS.std., SS.cri.) and SS.HU. were compared between anticoagulants used. In serum, VEGF levels were 151±17 in control (n=16), 517±76 in SS.std. (n=10), 476±40 in SS.cri. (n=4) and 463±109 in SS.HU.(n=4) respectively. In EDTA plasma were 22±2.6 in control (n=20), 115.4±51.6 in SS.std. (n=8), 73.4±9.9 in SS.cri. (n=8) and 170.0±53.3 in SS.HU. (n=7) respectively. In CTAD plasma were 10.7±0.4 in control (n=21), 18.0±1.1 in SS.std. (n=13), 23.0±3.3 in SS.cri. (n=7) and 23.5±3.6 in SS.HU. (n=8) respectively. (Figure 5.2.2)

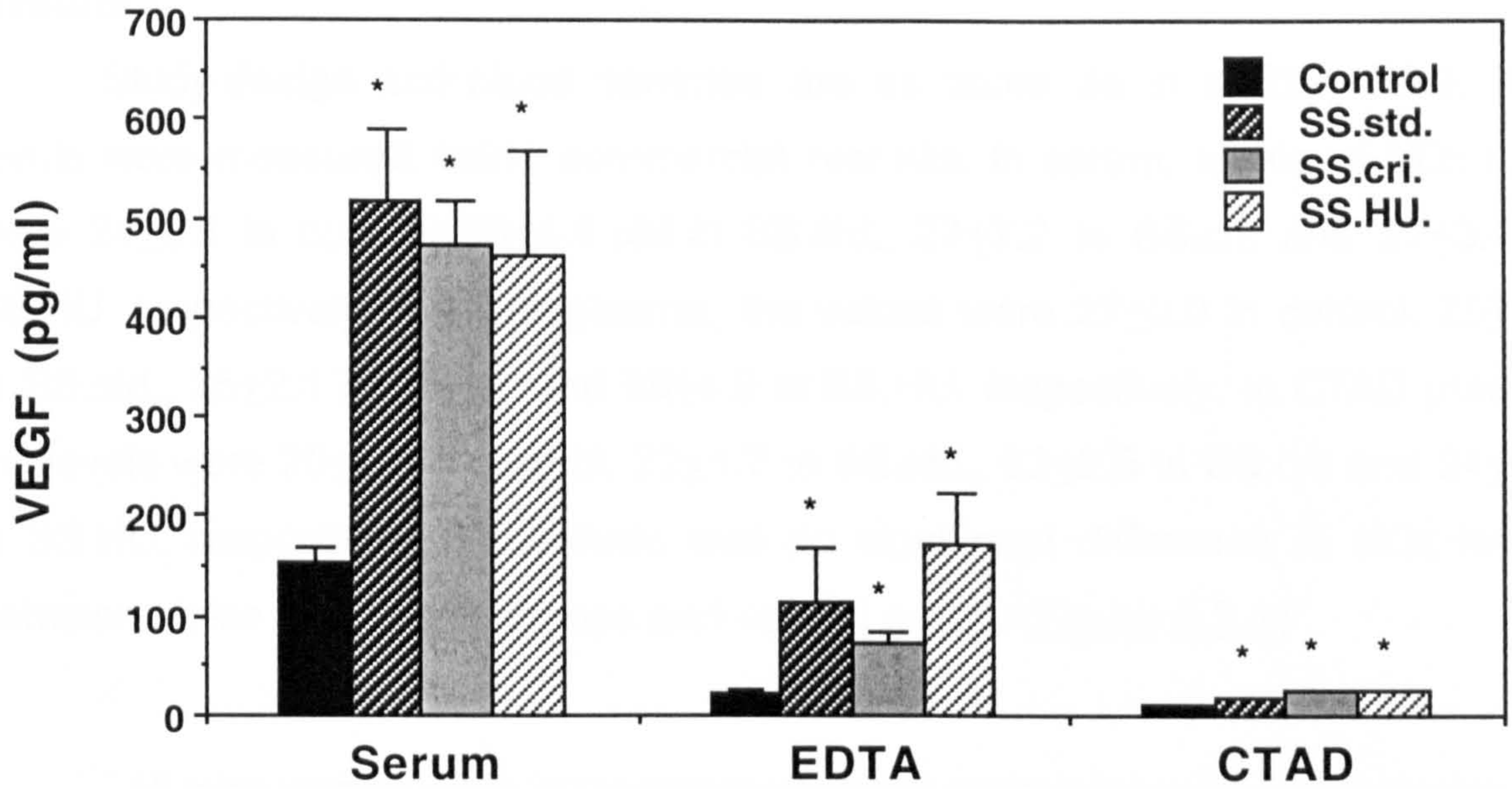


Figure 5.2.2 Levels of VEGF in samples from different anticoagulants

Serum samples of control subjects (n=16), sickle cell patients in steady state (SS.std., n=10), sickle cell patients in crisis (SS.cri., n=4) and sickle cell patients undergoing hydroxyurea treatment (SS.HU., n=4); EDTA plasma of control (n=20), SS.std. (n=8), SS.cri. (n=8) and SS.HU. (n=7) and CTAD plasma of control (n=21), SS.std. (n=13), SS.cri. (n=7) and SS.HU. (n=8) were assayed for VEGF (pg/ml) using commercial ELISA kits. *Indicates significantly higher than control group ($p<0.01$). There was no significant difference between 3 SS groups.

Discussion

VEGF levels in sickle cell patients were significantly higher than in control subjects in serum ($p<0.001$), EDTA ($p<0.01$) and CTAD ($p<0.001$). There were no significant differences between the 3 groups of sickle cell patients. Levels in serum were significantly higher than in EDTA and CTAD for both SS and control subjects. The higher values in serum could reflect in vitro platelet activation. Levels were further reduced in all patient types in CTAD, supporting this hypothesis. The effects of platelet activation on plasma VEGF are explored in subsequent sections.

5.2.3. NOx levels in blood samples from different anticoagulants

Results

Study design and blood samples are as same as in section 5.2.2. NOx levels were measured using commercial test kits. In serum, levels of NOx (μM) were 24±2.1 in control, 26±4.4 μM in SS.std., 27±7.2 in SS.cri. and 27±3.4 in SS.HU. respectively. In EDTA plasma, the values were 27±3.0 in control, 25±2.4 in SS.std., 25±2.1 in SS.cri. and 28±4.9 in SS.HU. respectively. In CTAD plasma the levels were 20±1.5 in control, 22±1.7 in SS.std., 23±2.9 in SS.cri. and 24±2.4 in SS.HU. respectively. Thus there was no significant difference in NOx levels between sickle cell patient groups and control group. (Figure 5.2.3)

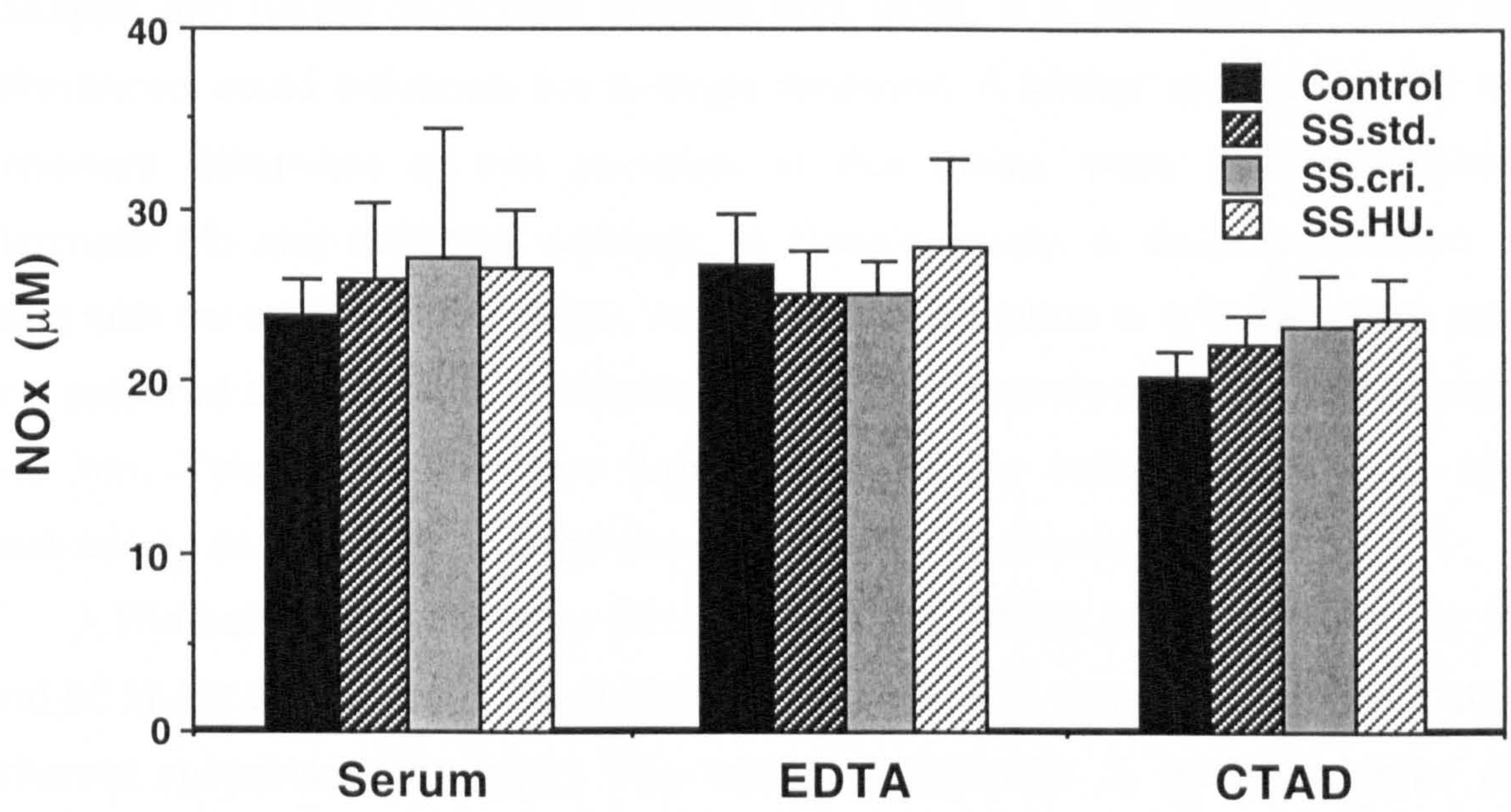


Figure 5.2.3 Levels of NOx in samples from different anticoagulants

Serum samples of control subjects (n=16), sickle cell patients in steady state (SS.std., n=10), sickle cell patients in crisis (SS.cri., n=4) and sickle cell patients undergoing hydroxyurea treatment (SS.HU., n=4); EDTA plasma of control (n=20), SS.std. (n=8), SS.cri. (n=8) and SS.HU. (n=7) and CTAD plasma of control (n=21), SS.std. (n=13), SS.cri. (n=7) and SS.HU. (n=8) were assayed for NOx concentration (μM) using commercial Griess reaction test kits. There was no significantly difference.

Discussion

The lack of difference in NOx levels between SS and control subjects contrasts with the findings of Rees *et al*, 1995, who found raised levels, particularly in SS.cri. However Lopez *et al*, 1996 reported that SS.cri. patients did not always show raised NOx levels. They separated SS.cri. patients into 3 groups according to pain score measured by a 10-cm visual analog scale (VAS) and found that high pain score was associated with low levels of NOx and vice versa. However, the method used by Rees was colorimetry and by Lopez was chemiluminescence. In this thesis colorimetry was used but there were two differences from Rees's method. Firstly NOx levels in lithium-heparin plasma samples were assayed by Rees and using Cadmium as the nitrate reductant, whereas in this thesis 3 types of sample (serum, EDTA and CTAD plasma) were assayed and nitrate reductase enzyme was used. It is not clear whether these differences could influence the findings however. A further and potentially more important difference is that samples in this thesis were pre-ultrafiltrated to eliminate Hb and bilirubin, whereas in Rees's study, a de-proteinisation was used with no methodology detail. As the Griess reaction is a colorimetric assay, any coloured substance may interfere with the endpoint absorbance especially at 540 nm. This could therefore have led to falsely increased in NOx levels, particularly as SS crisis is often associated with increased jaundice.

The same patients were not compared in steady state and in crisis or on and off HU in this section. It is therefore possible that small differences between different subjects could occur. The timing of samples in vaso-occlusive crisis may also influence NOx levels. A recent report showed that NOx levels were lower in patients who had experienced more and longer pain (Lopez *et al*, 2000). Samples taken in vaso-occlusive crisis in this thesis were all taken from patients who had experienced pain for more than 1day and therefore the reduced levels reported in early SS crisis by Lopez would not have been observed. Lopez suggested that the low levels of NOx seen in patients with prolonged vaso-occlusive pain could reflect a relative inability to increase NOx production in response to local hypoxia. It is possible that the patients reported by Lopez, who

made low NOx, are more likely to experience prolonged pain than those who produce higher levels. This is unproven at the present. Although sequential samples were not taken in crisis, the findings in this thesis provide no evidence to support the idea that NOx levels are significantly higher or lower than in control subjects, either in SS in crisis or in steady state.

5.2.4. VEGF and NOx levels in the same samples taken in different anticoagulants

Study design and results

In the previous sections, comparisons between VEGF and NOx levels obtained from serum, EDTA and CTAD plasma samples were not in the same subjects and not taken at the same time. Further experiments were therefore performed comparing measured levels of VEGF and NOx in serum, EDTA and CTAD plasma taken from the same healthy individuals. In 8 paired samples, the levels of VEGF in serum, EDTA and CTAD plasma were 131±23.4, 14±1.0 and 10±0.4 pg/ml respectively, confirming the findings of the previous section of significantly lower VEGF levels in EDTA and CTAD plasma than that in the serum ($p<0.005$) and the level in CTAD plasma was significantly lower than in EDTA plasma ($p<0.05$). The NOx levels in serum, EDTA and CTAD plasma were 24±2.5, 27±2.7 and 22±2.4 μM respectively, showing no significant difference (Figure 5.2.4).

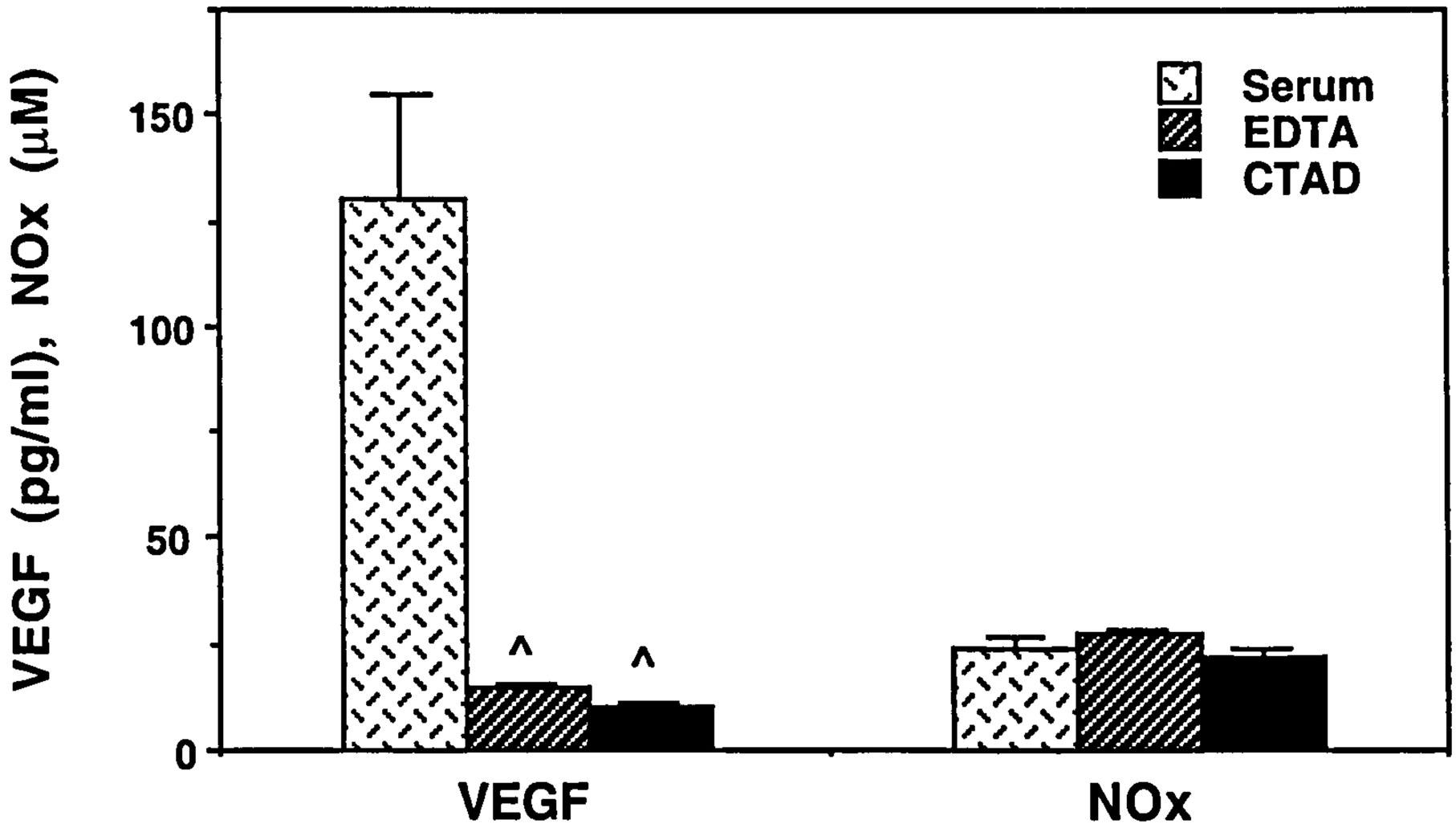


Figure 5.2.4 Levels of VEGF and NOx in paired samples using different anticoagulants

Eight paired samples from the same normal control subjects and taken at the same time but using different anticoagulants, were assayed for VEGF and NOx levels by commercial test kits. ^Indicates significantly lower than serum samples ($p<0.005$) and ^^ indicates significantly lower than EDTA plasma samples ($p<0.05$).

Discussion

These findings show that VEGF values are substantially higher in serum samples than in EDTA or CTAD plasma. The difference between VEGF in EDTA and CTAD plasma is less than that observed in the unpaired samples, but is still significantly higher in EDTA than in CTAD plasma ($p<0.05$). The NOx values are unaffected by the types of sample collection and anticoagulant used.

5.2.5. Effect of CTAD on ELISA assays for VEGF

Study design and results

In order to ensure that the lower VEGF levels seen in CTAD samples above were not due to an inhibitory effect of CTAD on the ELISA assay, six pairs of normal serum samples, with and without addition of CTAD anticoagulant at the same concentration as in the CTAD blood collection tubes, were assayed for VEGF (section 2.5.3).

The levels of VEGF in serum samples without and with addition of CTAD prior to the assay were 105.8±7.1 and 103.7±7.4 pg/ml, this was non-significantly different (Table 5.2.5).

Table 5.2.5 Comparison of VEGF levels without and with CTAD addition

Sample Number	VEGF without CTAD	VEGF with CTAD addition
1.	120	110
2.	98	99
3.	113	116
4.	76	70
5.	105	107
6.	123	120
Mean ± SE	105.8±7.1	103.7±7.4

Six serum samples regardless of diagnosis, were assayed for VEGF without and with addition of CTAD anticoagulant at the similar concentration of CTAD in the CTAD blood collection tube, in order to determine whether there is any effect of CTAD on ELISA assay of VEGF. The mean VEGF levels of both groups were not significantly different.

5.2.6. Discussion and conclusions

These findings show a significant increase in VEGF in sickle patients over control subjects irrespective of the anticoagulant used ($p<0.001$). However VEGF levels were substantially lower both in control subjects and in sickle cell patients when *in vitro* platelet activation was inhibited by CTAD ($p<0.05$). In paired control

serum, EDTA and CTAD plasma samples (section 5.2.4), VEGF in serum is 131 pg/ml compared to 14 pg/ml in CTAD representing nearly a 10 fold difference. This large difference is not due to inhibition of the assay by CTAD and must represent a difference resulting from inhibition of platelet activation *in vitro* by CTAD. This has been previously described and suggested that the majority of VEGF levels reported in the literature using serum were measuring platelet derived VEGF that was liberated *in vitro*, after sample collection (Adams *et al.*, 2000; Maloney *et al.*, 1998; Salgado *et al.*, 1999; Salven *et al.*, 1999; Verheul *et al.*, 1997; Webb *et al.*, 1998). It is clear from the results in this section that *in vitro* platelet activation contributes to the majority of measured VEGF in serum and EDTA plasma and such an effect is likely to swamp small differences in the blood levels of VEGF *in vivo*.

The differences between VEGF levels in SS and control subjects are seen in all types of sample collection. The question arises as to whether the higher VEGF in CTAD plasma reflects endothelial activation or residual increased platelet activation *in vivo*. It must be remembered that the platelet count in sickle cell patients is often higher than control subjects and this could lead to increased VEGF levels.

There is no evidence for differences in NOx between SS and control subjects. Furthermore, levels are not different between patients in crisis and in steady state.

5.3. EFFECT OF PLATELET ACTIVATION *IN VITRO* ON VEGF, NOx AND OTHER MARKERS

Rationale

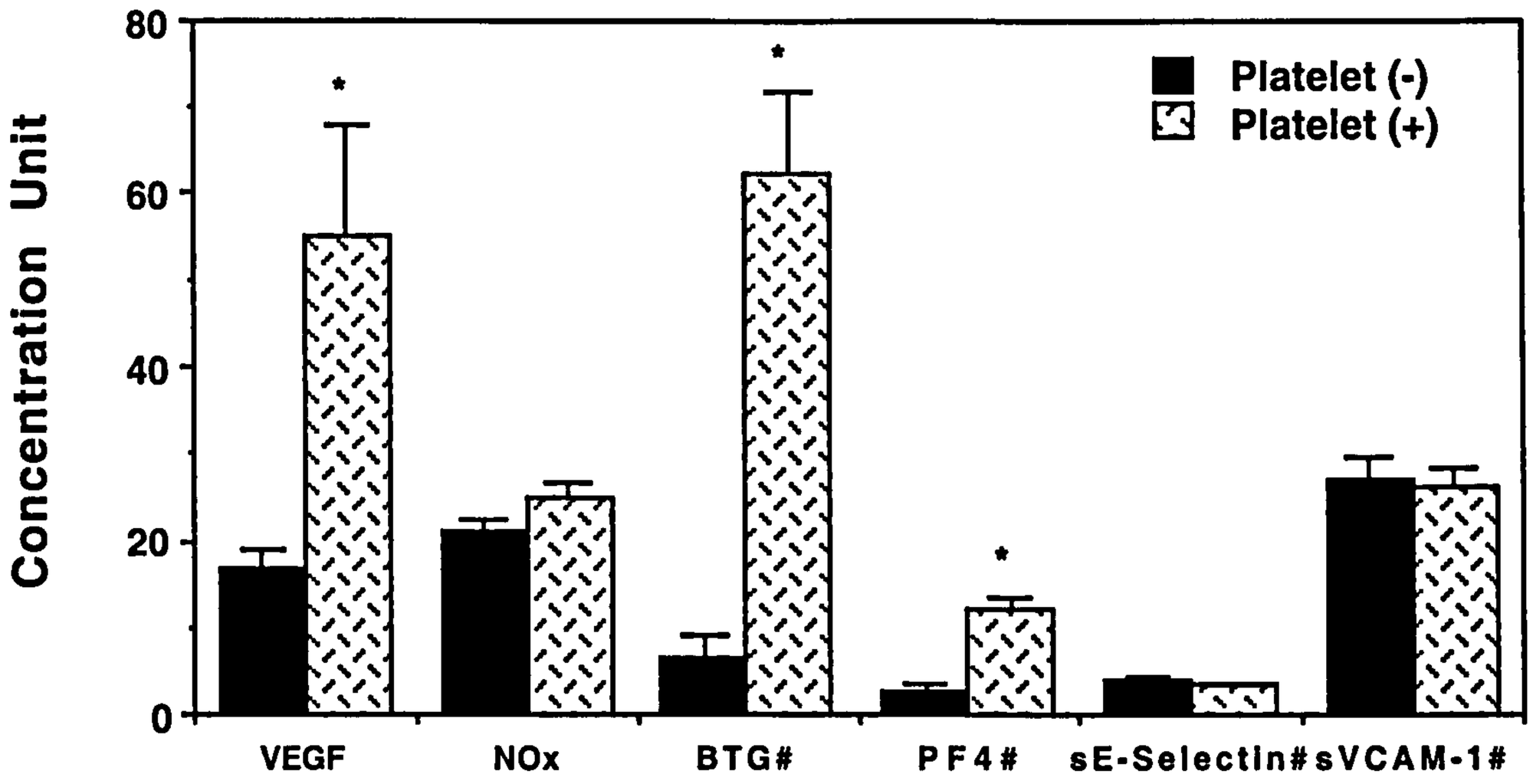
The findings in section 5.2 suggest that *in vitro* platelet activation may contribute to measured levels of VEGF, especially when the sample are not taken into CTAD anticoagulant. In this section, it was decided deliberately to induce platelet activation, using thrombin activating protein (TRAP), to determine the effect on VEGF and NOx and to compare this with known markers of platelet activation, such as beta-thromboglobulin (BTG) and platelet factor 4 (PF4). Markers considered to be specific for endothelial activation, soluble endothelial selectin (sE-selectin) and soluble vascular cell adhesion molecule 1 (sVCAM-1), were included as negative controls.

Study design

Six citrate platelet rich plasma samples from sickle cell patients were collected and deliberately activated using thrombin activating protein (TRAP) (section 2.5.5). In this experiment, citrate plasma was used instead of CTAD plasma because CTAD was designed for preventing platelet activation and such activation could not be performed. CTAD also contains citrate as anticoagulant together with theophylline, adenosine and dipyridamole for preventing platelet activation. There is no evidence of any interferace by citrate or CTAD on the ELISA assay as shown in section 5.2.5.

Results

The levels of VEGF without and with platelet activation *in vitro* (n=6) were 17±1.8 and 55±12.8 pg/ml ($p<0.03$); of NOx were 21±1.6 and 25±1.8 μM (non-significance; NS); of BTG were 68±23.4 and 626±90.0 IU/ml ($p<0.002$); of PF4 were 24±10.0 and 121±12.0 IU/ml ($p<0.003$); of sE-Selectin were 39.5±3.4 and 33.7±2.6 ng/ml (NS); of sVCAM-1 were 271±23 and 264±20 ng/ml (NS) respectively (Figure 5.3).



<u>Figure 5.3</u> Comparison of parameters from samples without and with platelet activation

Six paired citrate plasma samples without (-) and with (+) platelet activation were assayed for VEGF (pg/ml), NOx (μM), BTG (x10 IU/ml), PF4 (x10 IU/ml), sE-Selectin (x10 ng/ml) and sVCAM-1 (x10 ng/ml) using commercial test kits. * Indicates significantly higher than without platelet activation samples ($p<0.03$). # Indicates x10 of ordinary units.

Discussion

In platelet activated samples, VEGF, BTG and PF4 levels and BTG/PF4 ratio were significantly increased over their values in the samples without platelet activation. However neither NOx, sE-selectin nor sVCAM-1 levels were significantly changed by platelet activation. This confirms that platelet activation plays an important role in modulating measured VEGF serum levels and that platelets are a rich source of VEGF. The increase in citrate plasma VEGF following platelet activation (about 3 fold) is less than that seen with BTG (about 9 fold) or PF4 (about 5 fold). This suggests that platelets are an important source of serum VEGF, (because in serum, platelets are activated by the clotting process), but other sources are more likely to contribute to plasma VEGF levels. Human megakaryocytes are known to produce and secrete VEGF in an inducible

manner and purified megakaryocytes express mRNA of the three VEGF isoforms (121, 165, and 189 amino acids) (Mohle *et al.*, 1997). The presence of VEGF within platelets has implications for processes involving platelet and endothelial cell interactions. e.g. wound healing, and in tumor metastasis (Banks *et al.*, 1998). Platelet aggregation and thrombosis at the site of vessel injury secrete VEGF into that microenvironment, thus promoting neovascularisation. It is now known that VEGF is also secreted from platelets during the *in vitro* aggregation of platelet-rich plasma induced by thrombin, collagen, epinephrine, and ADP (Maloney *et al.*, 1998). It is not yet known whether activated platelets secrete all of their VEGF content or only part of it. On the other hand, levels of VEGF in citrate plasma, even after compensating for the slight dilution in addition of TRAP, seem to be lower than in serum samples. It is possible that some of the VEGF in serum samples results from activation of leukocytes (Salven et al, 1999) such as monocytes (Xiong, 1998) and neutrophils (Webb, 1998). It is clear that plasma VEGF especially CTAD plasma (with minimal platelet activation *in vitro*) gives a better reflection of *in vivo* levels than serum VEGF. As a result of this finding, it was decided to use CTAD plasma as the preferred measure of plasma VEGF.

5.4. RELATIONSHIPS OF VEGF AND NOx IN CTAD PLASMA

Blood samples

In this section and in subsequent sections through section 5.10 except section 5.9, the same group of patients with sickle cell disease and control subjects were studied. Their clinical and haematological data are shown below in Table 5.4.1.1 and Table 5.4.1.2.

Table 5.4.1.1 Clinical data of normal healthy control subjects

Number	Age	Sex	Crisis day	HU	WBC	Plt	RBC	Hb	HbF
Normal control donors (n=21)									
1.	36	M	-	-	6.8	277	4.71	14.5	0.4
2.	24	M	-	-	5.1	193	4.75	14.8	0.0
3.	26	F	-	-	6.7	292	4.33	12.1	0.8
4.	27	M	-	-	7.5	234	4.47	13.1	0.6
5.	36	F	-	-	7.9	171	4.96	13.9	0.1
6.	28	M	-	-	8.4	222	4.92	14.2	0.5
7.	39	M	-	-	7.2	198	5.44	15.0	0.4
8.	27	F	-	-	9.6	187	4.91	13.6	0.5
9.	30	F	-	-	6.1	297	4.26	13.4	0.3
10.	29	F	-	-	4.2	305	4.15	13.4	0.9
11.	39	M	-	-	8.6	245	5.20	16.4	0.6
12.	35	M	-	-	9.1	192	4.77	13.9	0.4
13.	27	M	-	-	6.7	170	5.22	15.2	0.9
14.	26	M	-	-	6.1	256	5.17	14.7	0.7
15.	25	F	-	-	5.8	208	4.25	13.2	0.1
16.	28	M	-	-	7.5	227	5.55	15.1	0.6
17.	24	F	-	-	5.8	203	4.44	12.0	0.4
18.	30	F	-	-	5.5	177	4.16	12.5	0.9
19.	40	M	-	-	8.5	215	4.94	14.0	0.8
20.	45	M	-	-	3.7	139	4.75	12.7	1.0
21.	31	M	-	-	7.8	220	4.67	13.0	1.0
Mean	31	13/8			6.9	220	4.76	13.8	0.6
SEM	1.3	(M/F)			0.3	9.8	0.09	0.25	0.07

Age (Years) Sex (M=male, F=Female) HU=on hydroxyurea
WBC ($10^3/mm^3$) Plt ($10^3/mm^3$) RBC ($10^6/mm^3$) Hb (g/dl) HbF (%)

Table 5.4.1.2 Clinical data of sickle cell patients

Number	Age	Sex	Crisis day	HU	WBC	Plt	RBC	Hb	HbF
Sickle cell patients in steady state (n=13)									
1.	9	M	-	-	15.5	381	1.82	6.0	3.5
2.	30	F	-	-	5.6	282	3.89	11.8	2.5
3.	23	M	-	-	16.7	455	3.85	10.7	6.2
4.	44	F	-	-	10.5	345	2.23	6.7	1.4
5.	8	F	-	-	14.1	322	2.66	8.0	5.2
6.	10	F	-	-	9.0	233	3.45	9.5	9.8
7.	26	M	-	-	14.9	207	3.37	9.9	7.0
8.	20	M	-	-	10.0	387	3.82	9.8	3.4
9.	31	F	-	-	7.8	295	2.20	6.7	4.0
10.	47	M	-	-	7.6	253	2.53	8.1	8.2
11.	23	F	-	-	9.9	317	2.73	8.7	9.6
12.	33	M	-	-	10.6	581	3.41	9.0	1.1
13.	57	M	-	-	7.8	174	2.18	6.6	1.6
Mean	28	7/6			10.8	326	2.93	8.6	4.9
SEM	4.2	(M/F)			0.96	30.3	0.20	0.5	0.8
Sickle cell patients in crisis (n=7)									
14.	25	M	1	-	10.6	146	3.59	7.7	7.0
15.	28	F	37	-	5.8	233	3.78	8.4	1.8
16.	26	F	3	-	9.4	230	2.21	6.8	3.3
17.	26	M	3	-	22.3	147	3.05	8.8	4.1
18.	51	F	7	-	12.2	326	2.32	7.3	7.6
19.	32	M	6	-	15.6	308	2.85	7.9	2.9
20.	30	M	10	-	11.7	454	2.06	5.2	3.8
Mean	31	4/3			12.5	263	2.84	7.4	4.4
SEM	43.4	(M/F)			1.9	41.3	0.26	0.5	0.8
Sickle cell patients on hydroxyurea (n=8)									
21.	23	F	-	Yes	12.1	528	2.74	8.4	16.2
22.	46	F	-	Yes	5.5	113	3.35	8.9	9.0
23.	33	F	31	Yes	6.0	342	2.04	7.1	11.0
44.	33	M	1	Yes	9.1	242	1.99	9.1	20.3
25.	57	M	-	Yes	4.5	186	3.13	10.2	17.8
26.	23	F	-	Yes	17.5	547	2.34	7.3	17.6
27.	35	M	-	Yes	8.4	210	2.88	9.8	23.7
28.	13	M	-	Yes	10.6	574	2.60	7.5	27.3
Mean	33	4/4			9.2	342	2.63	8.5	18.5
SEM	4.9	(M/F)			1.5	64.7	0.17	0.4	2.5

Age (Years) Sex (M=male, F=Female) HU = on hydroxyurea
WBC ($10^3/mm^3$) Plt ($10^3/mm^3$) RBC ($10^6/mm^3$) Hb (g/dl) HbF (%)

Overall results

Levels of CTAD plasma VEGF, NOx, platelet activation markers (BTG and PF4), endothelial activation markers (sE-Selectin and sVCAM-1) and anaemic markers (Hb and Epo) in normal control, SS.std., SS.cri. and SS.HU are shown in Table 5.4.2.1 and Table 5.4.2.2.

Table 5.4.2.1 Measured variables in normal healthy control subjects

Number	VEGF	NOx	BTG	PF4	sE-selectin	sVCAM-1	Epo
Normal Control Donors (n=21)							
1.	9	28	42	22	50	351	4
2.	12	13	15	6	33	391	5
3.	10	20	47	16	35	271	2
4.	17	29	36	13	37	351	3
5.	9	17	28	10	49	340	5
6.	12	20	23	7	39	317	5
7.	12	27	26	10	34	267	4
8.	10	20	16	8	54	380	6
9.	9	13	15	5	30	340	3
10.	8	19	27	11	31	350	4
11.	12	41	52	21	19	372	3
12.	10	26	21	8	49	312	4
13.	12	15	15	6	21	274	10
14.	11	12	24	8	28	291	5
15.	11	15	19	9	28	229	8
16.	10	19	13	5	49	330	5
17.	11	20	19	10	21	278	5
18.	9	15	11	4	27	388	2
19.	9	22	23	8	32	356	1
20.	10	18	46	15	40	409	5
21.	12	19	33	12	42	374	5
mean	11	20	26	10	36	332	4.5
SEM	0.4	1.5	2.6	1.1	2.2	10.6	0.4

VEGF (pg/ml), NOx (μM), BTG (IU/ml), PF4 (IU/ml), sE-selectin (ng/ml), sVCAM-1 (ng/ml), Epo (mIU/ml),

Table 5.4.2.2 Measured variables in sickle cell patients

Number	VEGF	NOx	BTG	PF4	sE-selectin	sVCAM-1	Epo
Sickle cell patients in steady state (n=13)							
1.	18	21	114	39	133	1229	122
2.	12	11	92	27	42	301	16
3.	14	12	107	36	58	727	41
4.	15	31	110	24	98	924	52
5.	19	24	202	53	191	1022	60
6.	14	20	159	32	198	913	125
7.	23	24	229	52	58	517	54
8.	22	33	149	29	76	501	15
9.	14	26	156	63	62	770	73
10.	23	20	63	12	88	953	71
11.	20	20	170	40	75	391	28
12.	19	25	221	58	71	565	32
13.	21	21	121	38	32	942	119
mean	18	22	145	39	91	750	62
SEM	1.1	1.7	14.1	4.1	15.6	76.8	10.7
Sickle cell patients in crisis (n=7)							
14.	13	18	87	37	52	1004	52
15.	15	17	123	36	79	931	55
16.	18	25	150	18	38	575	74
17.	38	18	92	18	99	660	83
18.	22	39	168	99	178	588	40
19.	27	26	145	42	56	611	59
20.	28	20	225	74	40	845	53
mean	23	23	141	46	77	745	59
SEM	3.3	2.9	17.9	11.3	18.7	67.3	5.5
Sickle cell patients on hydroxyurea (n=8)							
21.	10	13	44	15	54	598	42
22.	17	20	19	4	39	825	93
23.	13	20	94	22	44	684	137
24.	21	32	146	30	61	903	60
25.	35	19	165	42	37	872	41
26.	23	25	210	54	78	572	38
27.	32	32	112	44	31	432	142
28.	37	28	231	60	73	583	89
mean	24	24	128	34	52	684	80
SEM	3.6	2.4	26.5	6.9	6.1	59.3	14.9

VEGF (pg/ml), NOx (μM), BTG (IU/ml), PF4 (IU/ml), sE-selectin (ng/ml), sVCAM-1 (ng/ml), Epo (mIU/ml),

Results

VEGF levels in CTAD in Figure 5.2.2 is not clearly shown because of the scale used. Therefore, it is shown again in Figure 5.4.1. The dot-plot relationship between VEGF and NOx levels in CTAD plasma was shown in Figure 5.4.2.

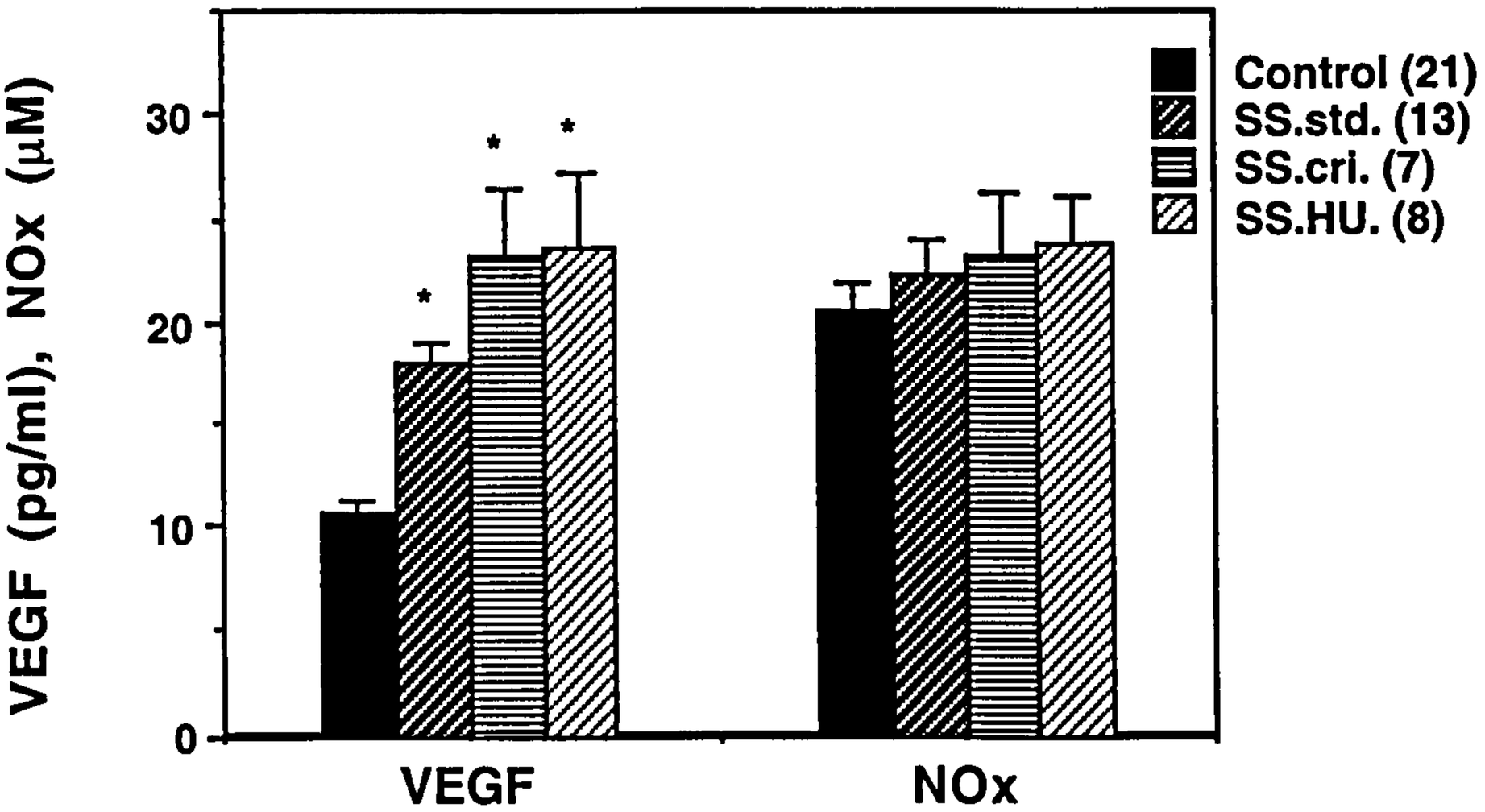
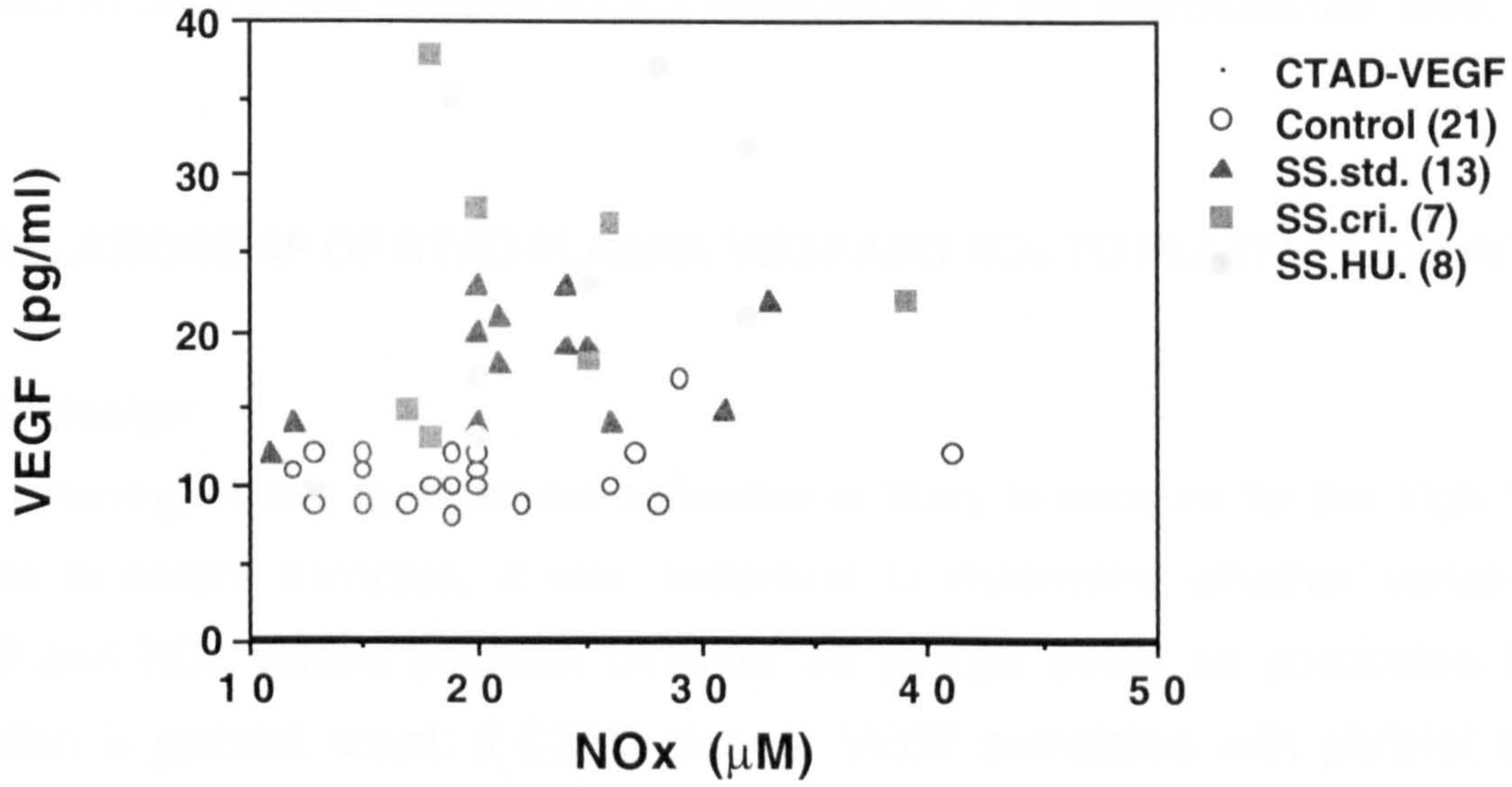


Figure 5.4.1 Comparison of CTAD plasma VEGF and NOx levels

A group of 21 normal control subjects, 13 sickle cell patients in steady state (SS.std.), 7 sickle cell patients in crisis (SS.cri.) and 8 sickle cell patients undergoing hydroxyurea treatment (SS.HU.) were assayed for VEGF (pg/ml), NOx (μM). *Indicates significantly higher than the control group ($p<0.001$).



<u>Figure 5.4.2</u> Dot-plot relationship between CTAD plasma VEGF and NOx levels

A group of 21 normal control subjects, 13 sickle cell patients in steady state (SS.std.), 7 sickle cell patients in crisis (SS.cri.) and 8 sickle cell patients undergoing hydroxyurea treatment (SS.HU.) were assayed for VEGF (pg/ml) and NOx (μM). There was no correlation between VEGF and NOx in any group of subjects. The correlation coefficient (r) were all lower than 0.50. The r values were 0.32 in all subjects together, 0.31 in control group, 0.40 in SS.std. group, 0.02 in SS.cri. group and 0.44 in SS.HU. group respectively.

Discussion

In <u>Figure 5.4.2</u>, it is clear that there is no correlation between VEGF and NOx in any patient group. Thus if hypoxia is a trigger for either VEGF and/or NO synthesis, this is not demonstrated by a change in these parameters at the same moment. This could be because the kinetics of changes in VEGF and NO synthesis and/or release are different. A primary increase in VEGF could lead to a secondary local increase in NO but this would occur several days later. Alternatively, NO synthesis may be modulated by factors independent of hypoxia and VEGF, which obscure changes associated with these molecules. Finally, for reasons already discussed, measurement of NOx in peripheral venous blood is unlikely to be an accurate indicator of changes at the microvascular level. These

observations, although not demonstrating a direct relationship between VEGF and NO in SS, cannot exclude such a relationship at the microvascular level.

5.5. RELATIONSHIP OF CTAD PLASMA VEGF AND NOx TO PLATELET COUNT

Study design

Having shown that platelet activation is likely to account for the high VEGF values in serum samples, it was important to determine whether variation in VEGF and NOx values between different SS groups could be accounted for by variation in platelet count. If CTAD plasma VEGF correlated with platelet count, this would suggest that plasma VEGF reflect platelet activation *in vivo*. If however plasma VEGF were independent of platelet count, this would suggest that other sources of plasma VEGF (e.g. endothelial VEGF) might account for the variability in plasma VEGF between the SS groups. CTAD plasma from controls (n=21), SS.std. (n=13), SS.cri. (n=7) and SS.HU. (n=8) were assayed for VEGF and NOx levels. These levels were then plotted against each other and against platelet count obtained from FBC.

Results

The mean platelet count ($x10^3/mm^3$) were 220±9.8 in controls, 326±30.3 in SS.std., 263±41.3 in SS.cri. and 343±64.7 in SS.HU. respectively. The dot-plot relationship between VEGF and platelet count was shown in Figure 5.5.1 and between NOx and platelet count in Figure 5.5.2.

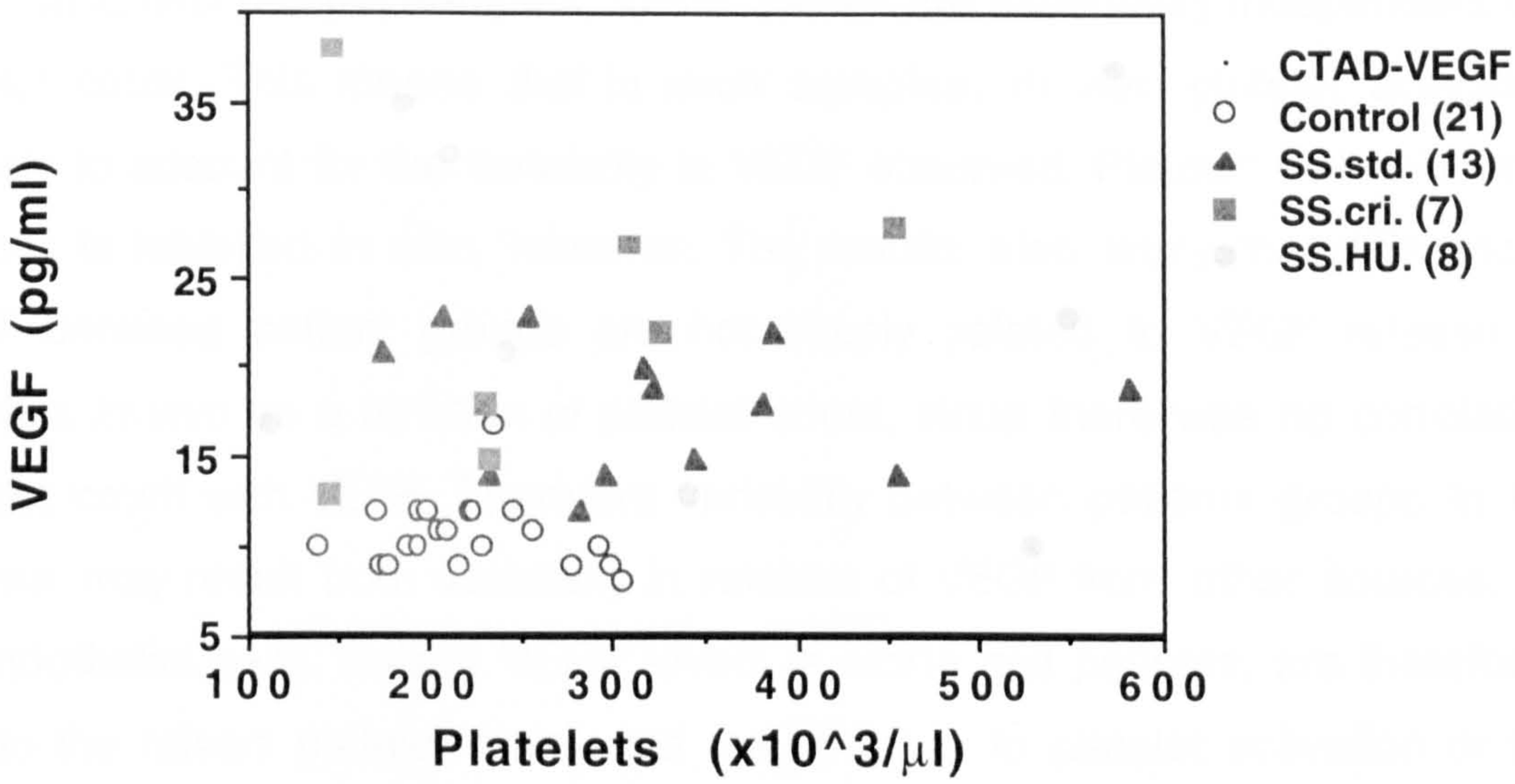


Figure 5.5.1 Dot-plot relationship between VEGF and platelet count

There was no correlation between VEGF and platelet count in CTAD plasma. The r values were 0.24 in all subjects together, 0.15 in control group, 0.15 in SS.std. group, 0.16 in SS.cri. group and 0.06 in SS.HU. group respectively.

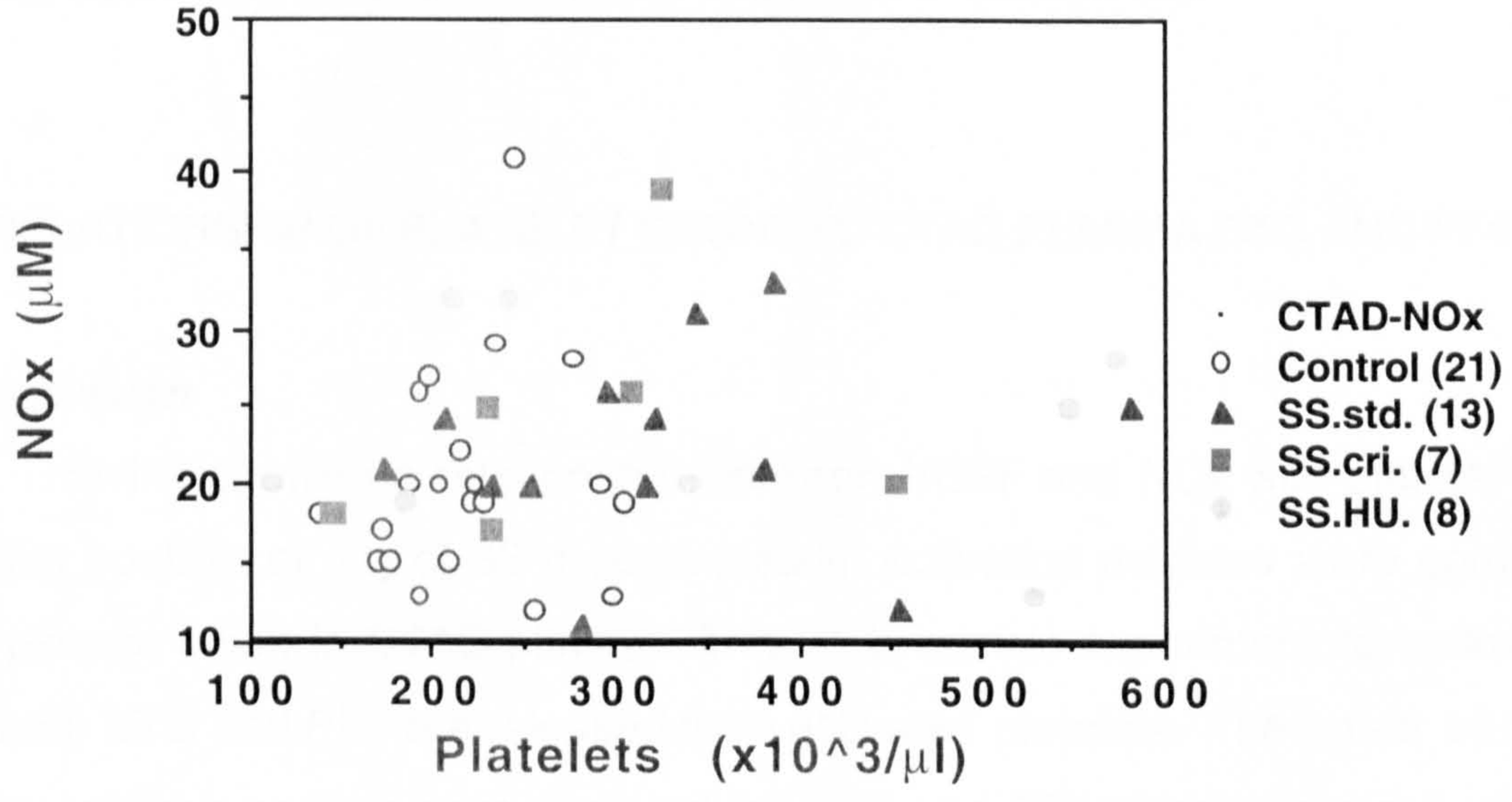


Figure 5.5.2 Dot-plot relationship between NOx and platelet count

There was no correlation between NOx and platelet count in CTAD plasma. The correlation coefficient (r) is less than 0.50 in all 4 groups of subjects. The r values were 0.15 in all subjects together, 0.14 in control group, 0.11 in SS.std. group, 0.36 in SS.cri. group and 0.12 in SS.HU. group respectively.

Discussion

In CTAD plasma samples, VEGF values are essentially independent of the platelet count. This means that in such samples, *in vitro* platelet activation is unlikely to account for the variability in VEGF observed. Platelet activation in this sample is inhibited *in vitro*, however. The results also imply that differences in VEGF between patient groups are not simply related to VEGF release from platelets *in vivo* as a function of platelet count, since there was no correlation of platelet count with VEGF. Therefore variability between patients groups in CTAD plasma may result from variability in release of VEGF from other sources, such as endothelial cells. Raised VEGF levels in sickle cell patients, are therefore not due to the raised platelet count, but may be due to platelet activation or to the VEGF release from other cells.

In order to address whether VEGF levels *in vivo* reflect platelet activation rather than platelet count. CTAD plasma VEGF levels were compared with values of two known platelet activation markers (BTG & PF4). However before this relationship was explored, preliminary examination of the relationship between platelet count and BTG and PF4 was examined in section 5.6.

5.6. RELATIONSHIP OF PLATELET COUNT TO CTAD PLASMA BTG AND PF4

Study design

Having shown no relationship between VEGF and NOx with platelet count in either controls or any of SS groups, platelet activation markers were compared with platelet count in CTAD plasma. This is a control experiment to make sure that both BTG and PF4 are released from activated platelets. The same samples as the previous section were assayed for BTG and PF4, then compared with the results of platelet count obtained from FBC.

Results and discussion

Platelet counts in normal controls, SS.std. SS.cri. and SS.HU. were 220±10, 326±30, 263±41 and 342±65 $x10^3/\mu l$ respectively. Levels of CTAD plasma BTG in control, SS.std., SS.cri. and SS.HU. were 26±2.6, 146±14.1, 141±17.9 and 128.4±26.5 IU/ml respectively and levels of PF4 were 10±1.1, 39±4.1, 46±11.3 and 34±6.9 IU/ml respectively (Figure 5.6.1). The dot-plot relationship between BTG and platelet count was shown in Figure 5.6.2, between PF4 and platelet count in Figure 5.6.3 and between BTG and PF4 in Figure 5.6.4.

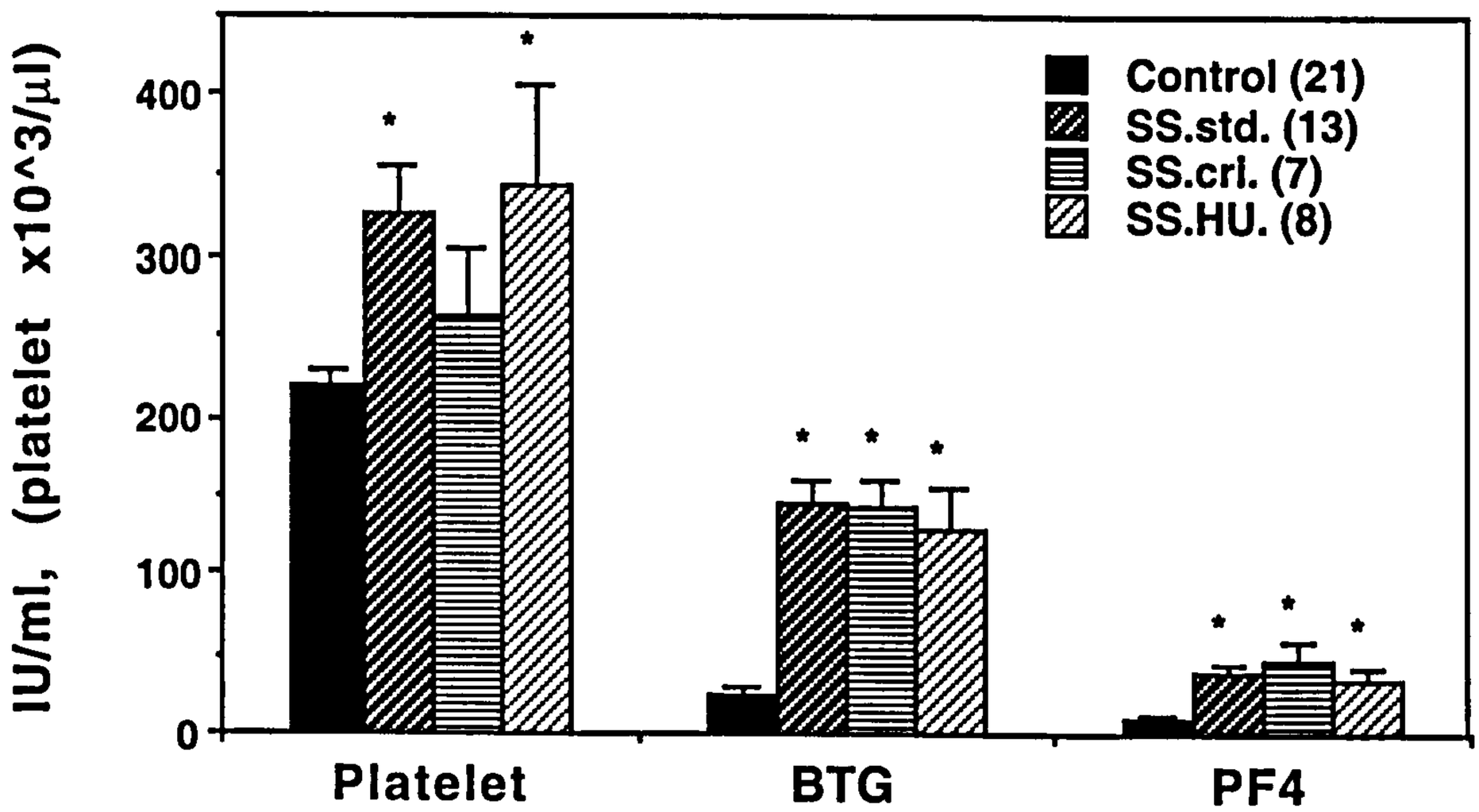


Figure 5.6.1 Comparison of platelet count and platelet activation markers

CTAD plasma from 21 normal control subjects, 13 sickle cell patients in steady state (SS.std.), 7 sickle cell patients in crisis (SS.cri.) and 8 sickle cell patients undergoing hydroxyurea treatment (SS.HU.) were assayed for platelet count ($x10^3/\mu l$), BTG (IU/ml) and PF4 (IU/ml). *Indicates significantly higher than the control group ($p<0.05$ for platelet count, <0.001 for the rest).

In all 3 groups of SS both BTG and PF4 were significantly higher than the control group ($p<0.001$). This shows a clear evidence of platelet activation *in vivo* in all 3 groups of SS. These levels in CTAD anticoagulant are likely to reflect the true levels *in vivo*. No significant difference was observed in either BTG or PF4 levels between the 3 groups of SS. However the platelet count was significantly

higher than control in SS.std. and SS.HU. but not in SS.cri. The fall in platelet count in SS.cri. relative to SS.std. in this thesis is in agreement with two previous studies (Alkjaersig *et al.*, 1976; Ittyerah *et al.*, 1976), suggesting more platelet consumption during the crisis. This could suggest that platelet activation and consumption in SS.cri. patients is very high.

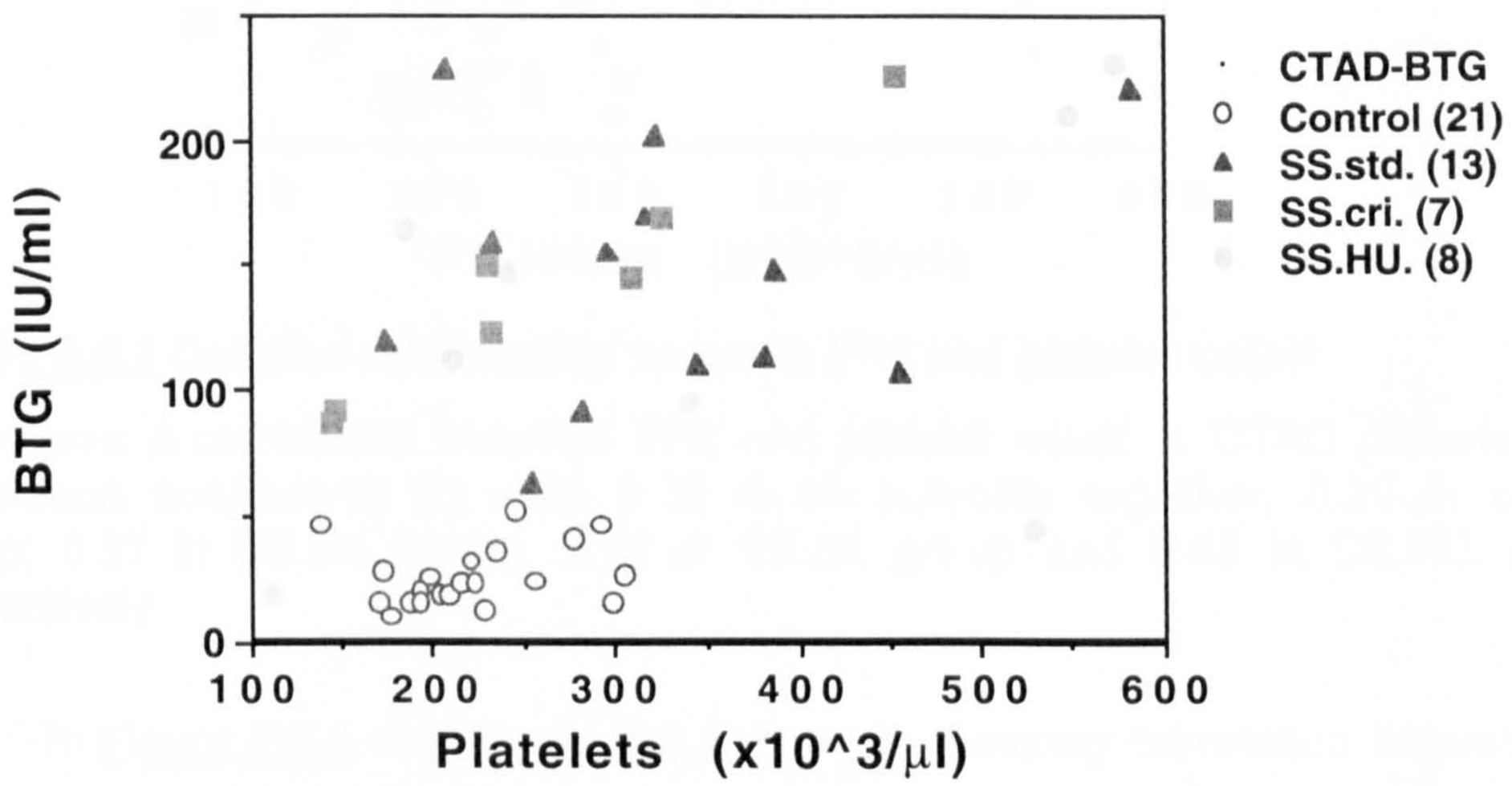


Figure 5.6.2 Dot-plot relationship between BTG and platelet count

The correlation coefficients (r) were 0.56 in all subjects together, 0.25 in control group, 0.18 in SS.std. group, 0.97 in SS.cri. group and 0.46 in SS.HU. group respectively.

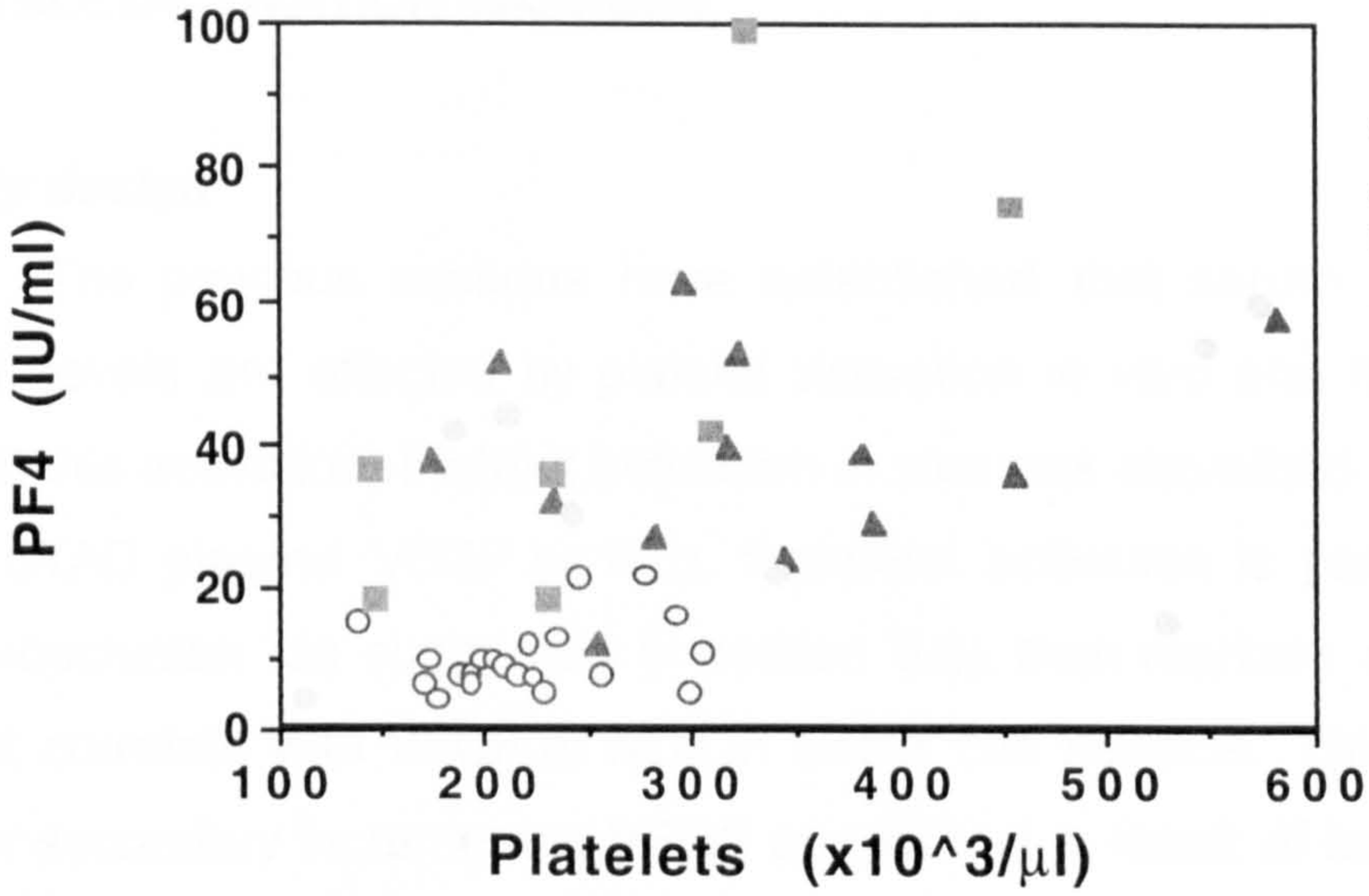


Figure 5.6.3 Dot-plot relationship between PF4 and platelet count

There was a correlation between PF4 and platelet count in CTAD plasma. The correlation coefficients (r) were 0.52 in all subjects together, 0.29 in control group, 0.37 in SS.std. group, 0.72 in SS.cri. group and 0.45 in SS.HU. group respectively.

In Figure 5.6.3 and Figure 5.6.4, there is a strong correlation between the platelet count and BTG (r=0.970) and PF4 (r=0.716) in SS.cri. Furthermore, these markers are significantly raised in SS.cri. (Figure 5.6.1). However, the platelet count in this particular group is actually lower, the raised levels of BTG and PF4 must reflect platelet activation. This finding is in agreement with previous reports that the platelets of crisis SS patients are easily activated *in vivo* and increased formation of aggregates and release of BTG and PF4 (Westwick *et al.*, 1983). In those reports, both the BTG and PF-4 levels were significantly higher in patients than in controls (Adamides *et al.*, 1990). In another study, ADP release indicated platelet activation during acute sickle pain crisis (Beurling-Harbury & Schade, 1989). Thus, the finding in this thesis confirms platelet activation in SS with high levels of platelet activation markers and platelet consumption in crisis patients.

5.7. RELATIONSHIP OF CTAD PLASMA VEGF AND NOx TO PLATELETACTIVATION MARKERS

Study design

The previous sections have established that serum and EDTA plasma VEGF levels are affected by platelet activation *in vitro* and that CTAD samples inhibit this activation. Platelet activation *in vivo* was examined for any relationship with CTAD plasma VEGF or NOx. If platelet activation is part of the process of vaso-occlusion (as suggested in section 5.6), then markers of platelet activation might correlate with VEGF or NOx in sickle cell patients. This could result from either secondary increment in VEGF and NO as a result of local hypoxia, or from release of VEGF from activated platelets. The same CTAD samples as in the previous section were used. VEGF and NOx levels were compared with BTG and PF4 levels.

Results

CTAD plasma VEGF (pg/ml) levels were 10.7±0.4 in controls (n=21), 18.0±1.1 in SS.std. (n=13), 23.0±3.3 in SS.cri. (n=7) and was 23.5±3.6 in SS.HU. (n=8) respectively. The CTAD plasma NOx (μM) levels were 20±1.5 in controls, 22±1.7 in SS.std., 23±2.9 in SS.cri. and 24±2.4 in SS.HU. respectively.

CTAD plasma levels of BTG in controls, SS.std., SS.cri. and SS.HU. were 26±2.6, 146±14.1, 141±17.9 and 128.4±26.5 IU/ml respectively and of PF4 were 10±1.1, 39±4.1, 46±11.3 and 34±6.9 IU/ml respectively.

The dot-plot relationship between VEGF and BTG is shown in Figure 5.7.1, between VEGF and PF4 in Figure 5.7.2, between NOx and BTG in Figure 5.7.3, between NOx and PF4 in Figure 5.7.4 and between BTG and PF4 in Figure 5.7.5.

For all samples, these appear to be a weak correlation between VEGF and BTG (r=0.69) and between VEGF and PF4 (r=0.58) respectively. In SS.HU. group alone, these correlation were also weak (r=0.71 and 0.79 respectively). In the another 2 groups, no correlation was observed. These weak correlation may be due to a slight contribution of platelet activation *in vivo*, which is known to release

some VEGF. Otherwise it may be a false correlation, because when all samples are put together, the lower value in controls and the higher values in patients might create an accidental correlation.

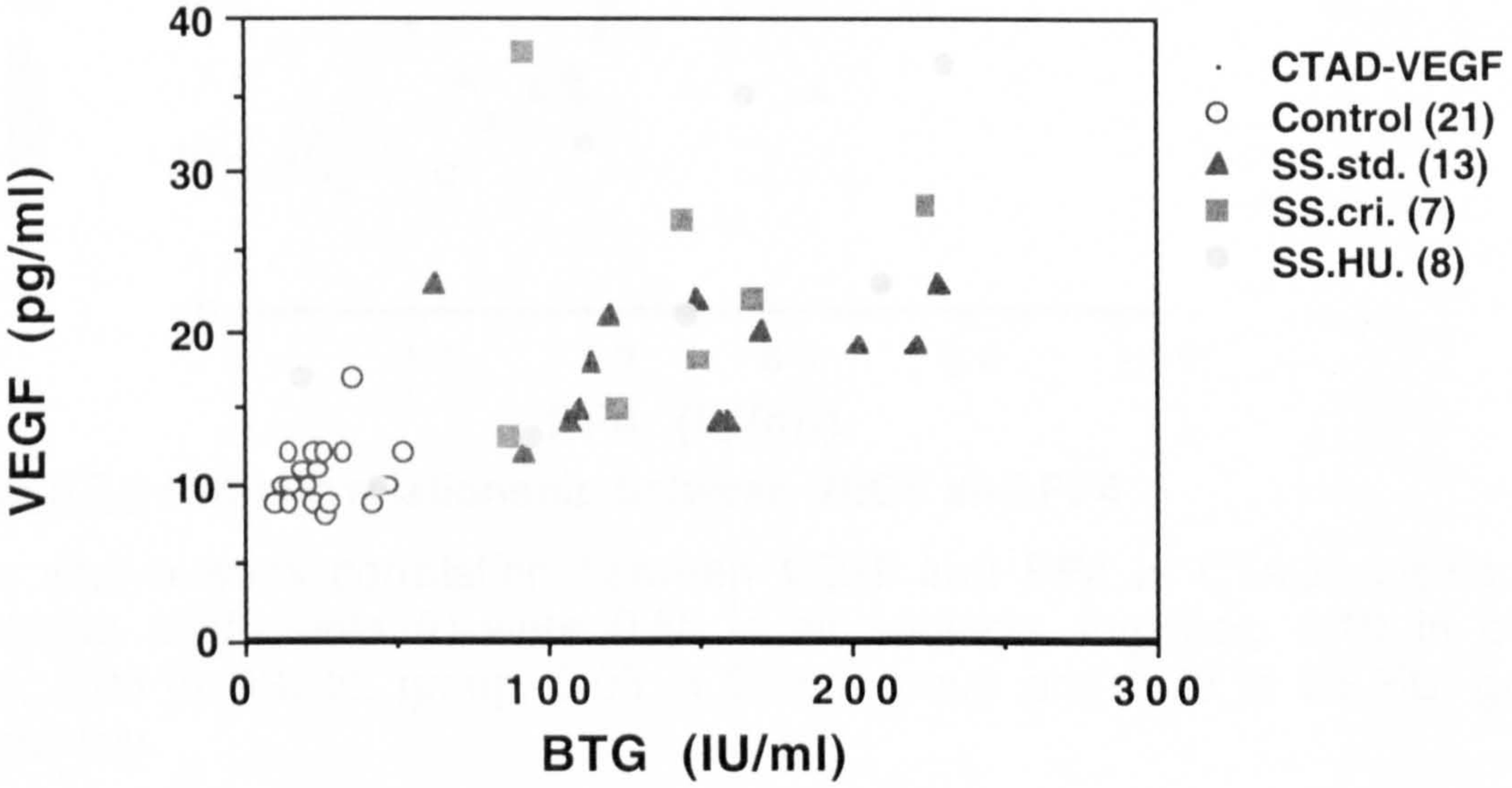


Figure 5.7.1 Dot-plot relationship between VEGF and BTG

There is a weak correlation between VEGF and BTG in CTAD plasma. The correlation coefficients (r) were 0.69 in all subjects together, 0.16 in control group, 0.27 in SS.std. group, 0.13 in SS.cri. group and 0.71 in SS.HU. group respectively.

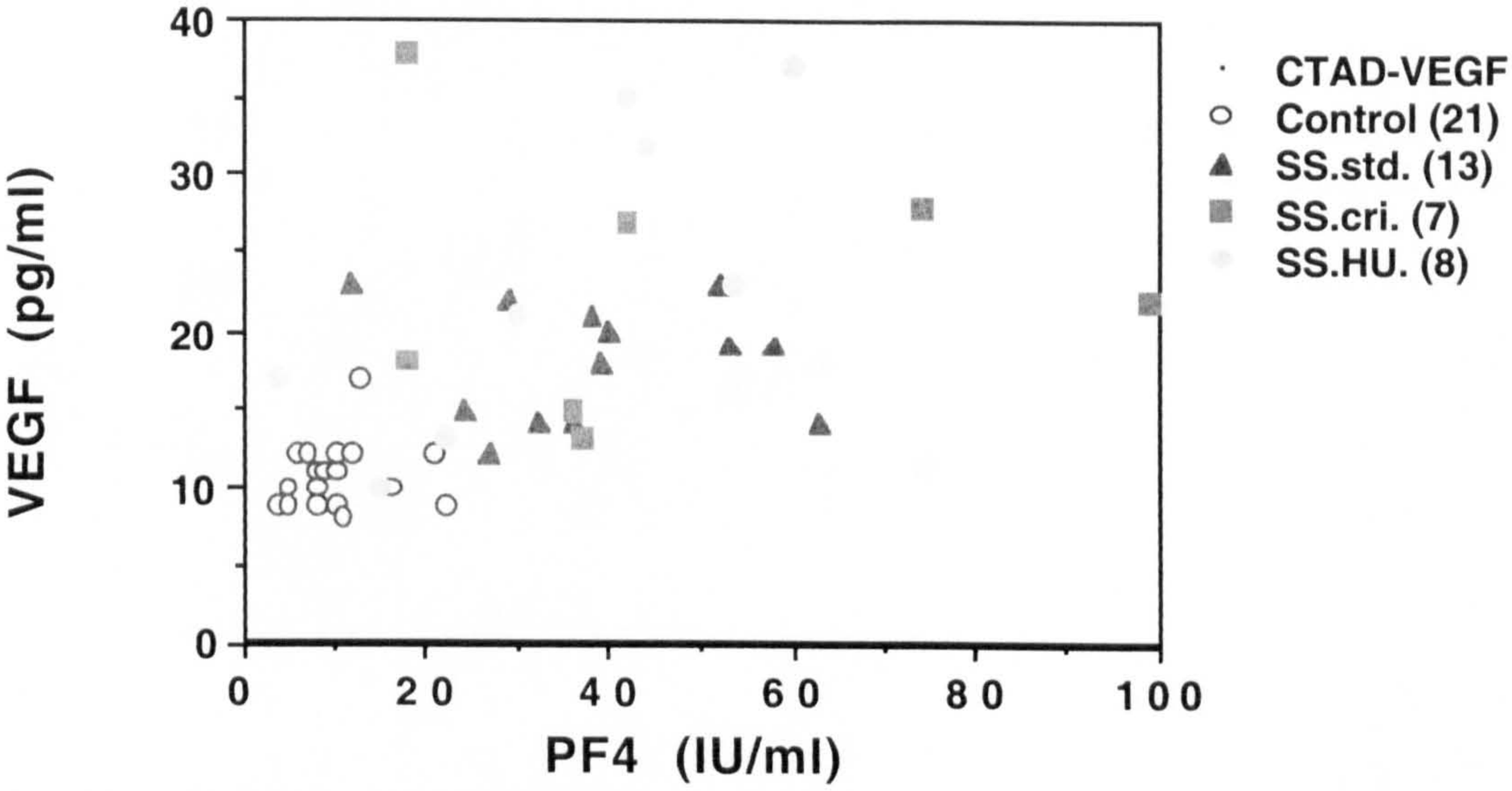


Figure 5.7.2 Dot-plot relationship between VEGF and PF4

There was a weak correlation between VEGF and PF4 in CTAD plasma. The correlation coefficients (r) were 0.58 in all subjects together, 0.10 in control group, 0.03 in SS.std. group, 0.03 in SS.cri. group and 0.79 in SS.HU. groups respectively.

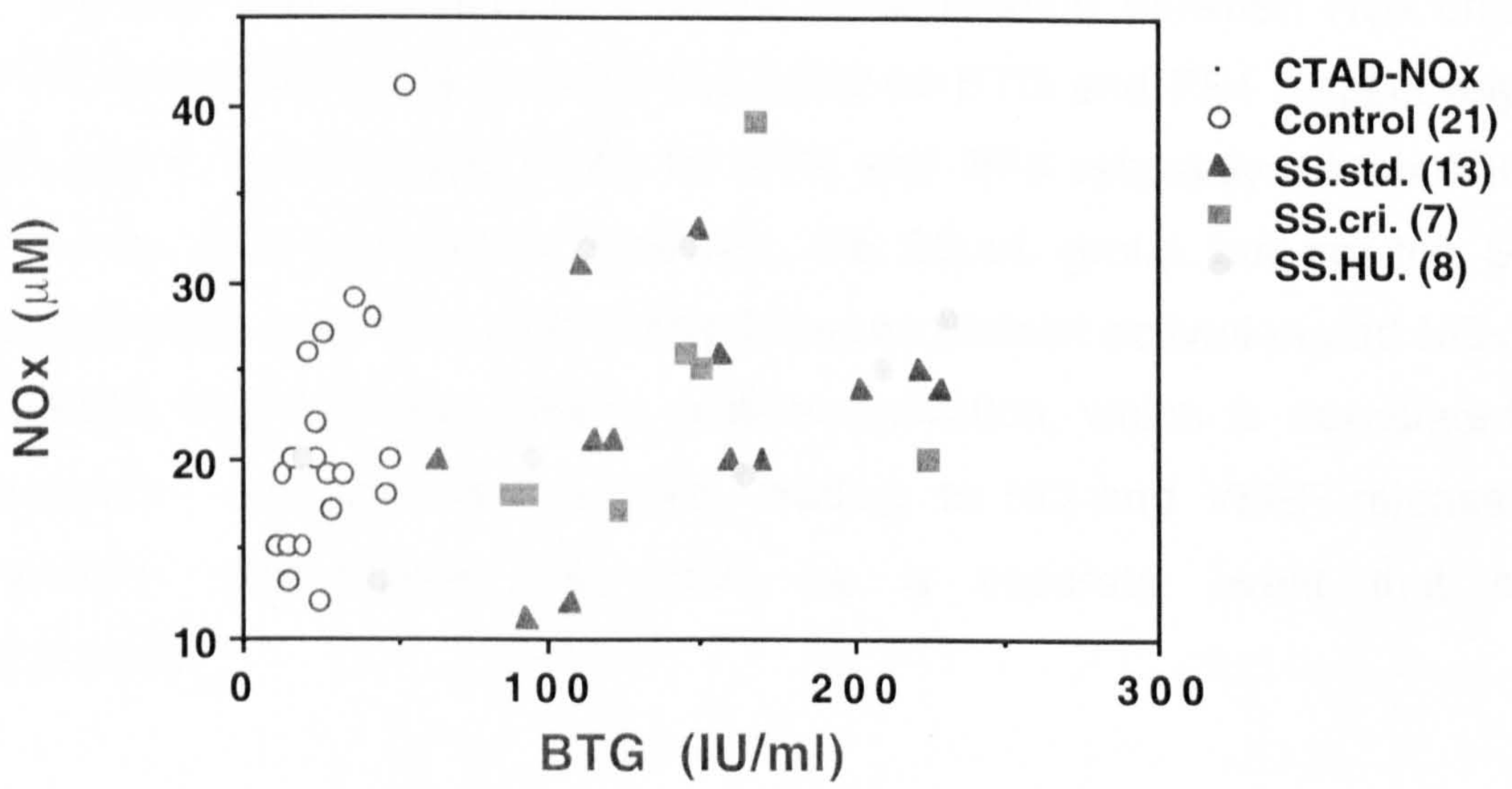


Figure 5.7.3 Dot-plot relationship between NOx and BTG

There was no correlation between NOx and BTG in CTAD plasma. The correlation coefficients (r) were 0.36 in all subjects together, 0.62 in control group, 0.37 in SS.std. group, 0.38 in SS.cri. group and 0.51 in SS.HU. group respectively.

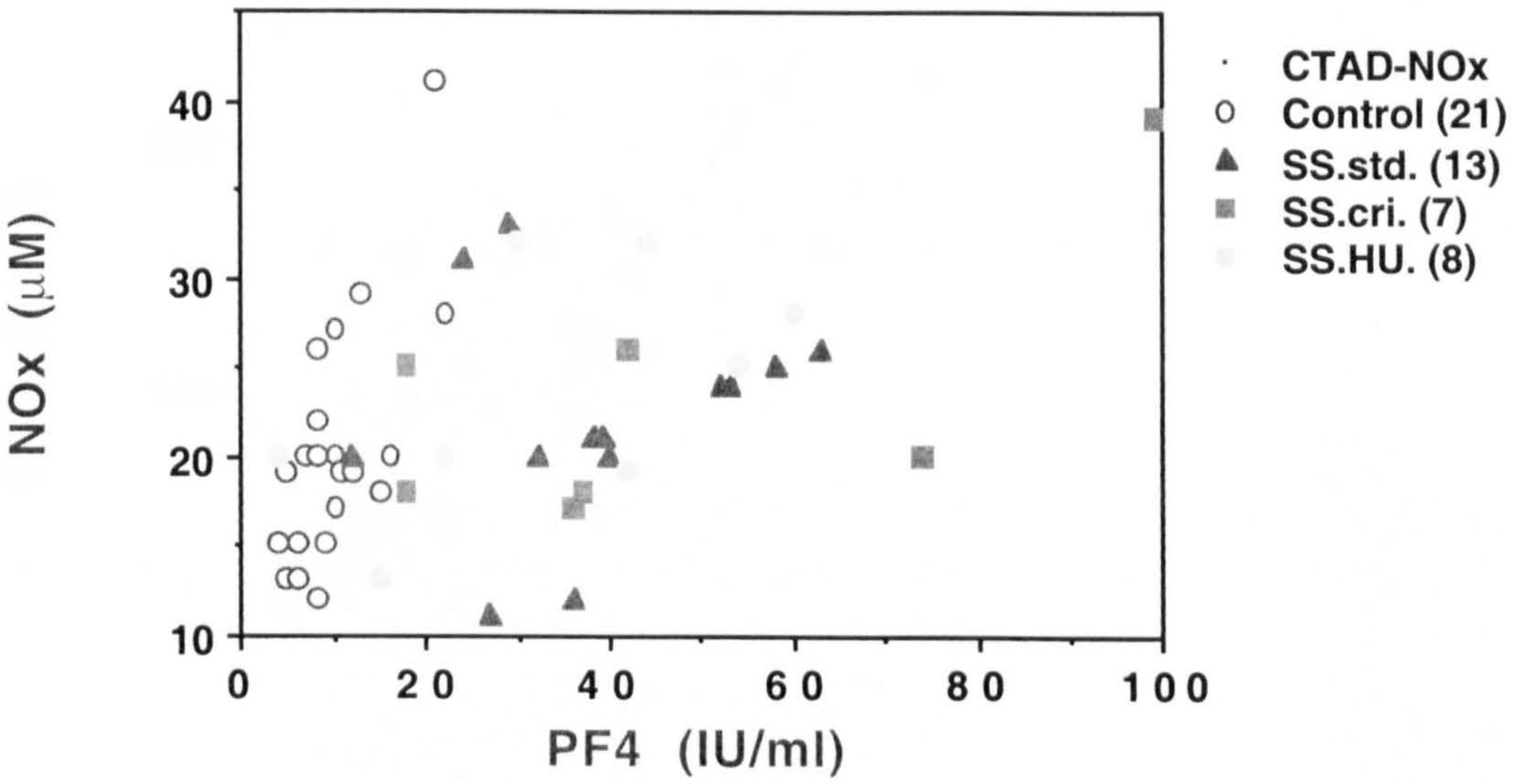


Figure 5.7.4 Dot-plot relationship between NOx and PF4

There was no correlation between NOx and PF4 in CTAD plasma. The correlation coefficients (r) were 0.43 in all subjects together, 0.69 in control group, 0.18 in SS.std. group, 0.67 in SS.cri. group and 0.55 in SS.HU. group respectively.

Although the overall data showed no correlation between NOx and BTG and PF4, the control group (r=0.620 and 0.692 for BTG and PF4 respectively) and SS.HU. group (r=0.511 and 0.550 for BTG and PF4 respectively) appeared to show some weak correlation. However, the SS.cri. group but not the SS.std. group, appeared to show a relationship between platelet activation and NOx. This could mean that in crisis, there is platelet activation, which is associated with microvascular occlusion and hypoxia, leading to NO and VEGF release from endothelium. Alternatively, this could be a separate event that occurs coincidentally.

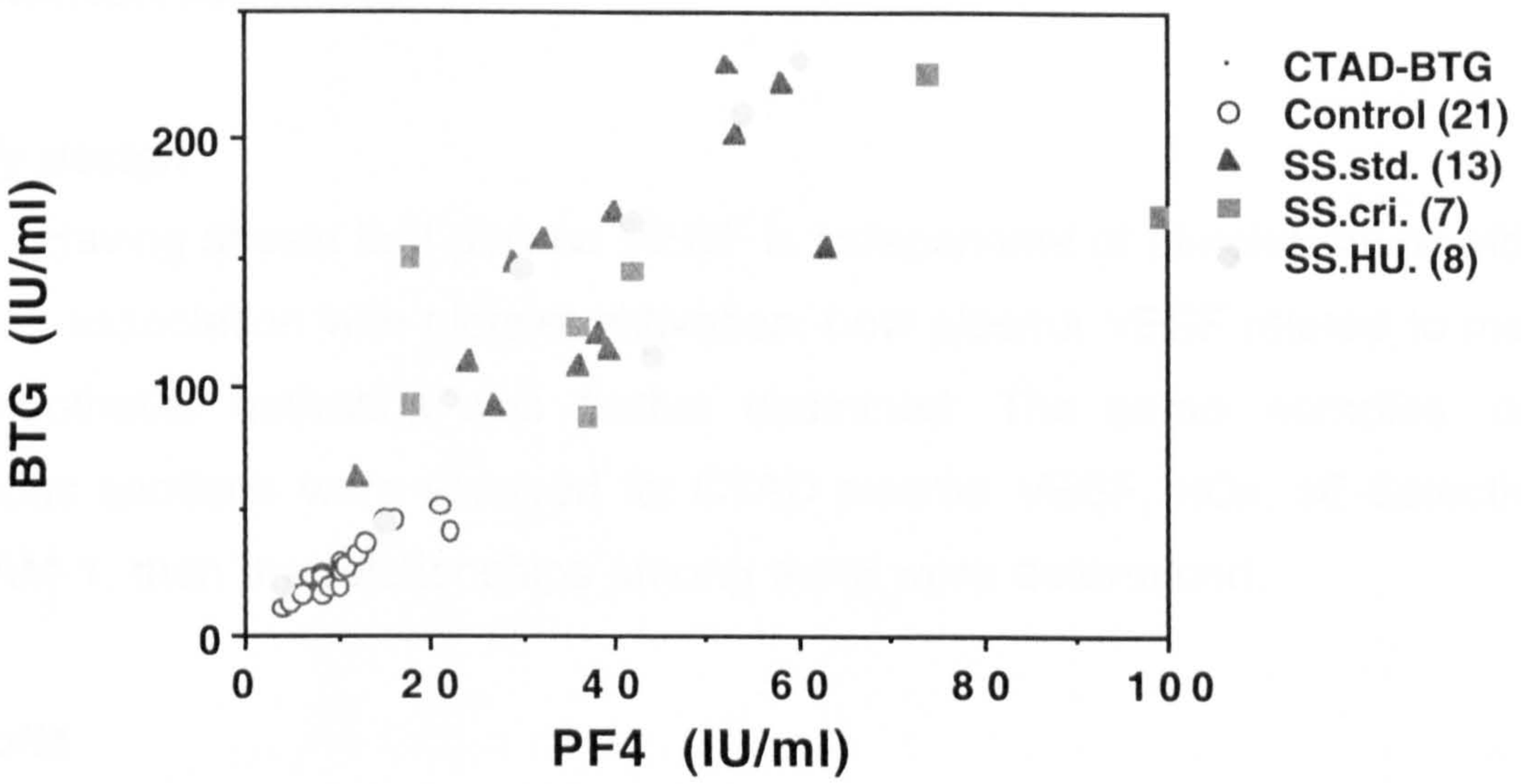


Figure 5.7.5 Dot-plot relationship between BTG and PF4

There was a strong correlation between BTG and PF4 in CTAD plasma. The correlation coefficients (r) were 0.87 in all subjects together, 0.93 in control group, 0.79 in SS.std. group, 0.65 in SS.cri. group and 0.94 in SS.HU. group respectively.

Discussion

The weak correlation between VEGF and platelet activation *in vivo* found in this section, is important because it contrasts with the lack of correlation between VEGF and platelet counts in the previous section. This suggests that plasma VEGF values are not derived totally from platelet activation in SS patients. CTAD plasma VEGF appears to be independent of platelet count but platelet activation still contributes at least in part to VEGF levels in SS patients. It is likely that VEGF values also reflect activation of other VEGF containing cells. In the next section, the relationship of VEGF to markers of endothelium activation is addressed to examine whether activation of these cells is important in determining VEGF levels. The strong correlation between BTG and PF4 is the consequence of their concomitant release from the same alpha-granules after platelet activation.

5.8. RELATIONSHIP OF CTAD PLASMA VEGF AND NOx TO ENDOTHELIAL ACTIVATION MARKERS

Study design

Having shown that plasma VEGF is independent of platelet count with only a weak association with platelet activation, how plasma VEGF related to markers of endothelial activation was further examined. The same samples as the previous sections were assayed for CTAD plasma VEGF, NOx, sE-Selectin and sVCAM-1, then the relationships among them were determined.

Results

Levels of CTAD plasma sE-Selectin in controls (n=21), SS.std. (n=13), SS.cri. (n=7) and SS.HU. (n=8) were 36±2.2, 91±14.6, 77±18.7 and 52±6.1 ng/ml respectively and of sVCAM-1 were 332±10.6, 750±76.8, 745±67.3 and 684±59.3 ng/ml respectively. This shows a significant increase of both sE-selectin and sVCAM-1 in all 3 SS groups over the control group ($p<0.001$), but no significant difference was observed between SS.std., SS.cri. and SS.HU. (Figure 5.8.1).

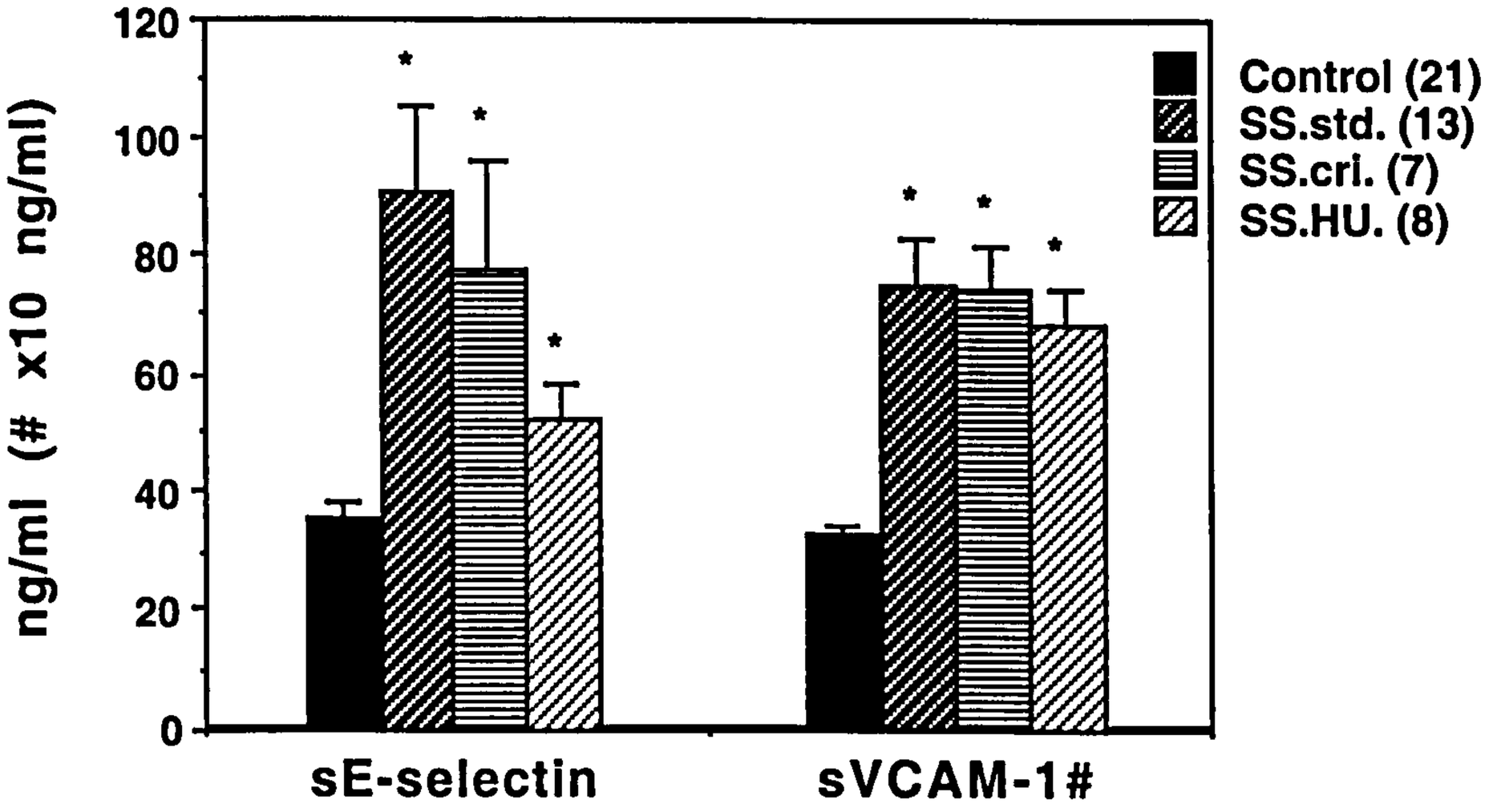


Figure 5.8.1 Comparison of endothelial activation markers

CTAD plasma from 21 normal control subjects, 13 sickle cell patients in steady state (SS.std.), 7 sickle cell patients in crisis (SS.cri.) and 8 sickle cell patients undergoing hydroxyurea treatment (SS.HU.) were assayed for sE-Selectin (ng/ml) and sVCAM-1 (x10 ng/ml). *Indicates significantly higher than control group ($p<0.005$). #Indicates x10 of actual values.

The dot-plot relationship between sE-Selectin and sVCAM-1 is shown in Figure 5.8.2, between VEGF and sE-Selectin is shown in Figure 5.8.3, between VEGF and sVCAM-1 in Figure 5.8.4, between NOx and sE-Selectin in Figure 5.8.5 and between NOx and sVCAM-1 in Figure 5.8.6 respectively.

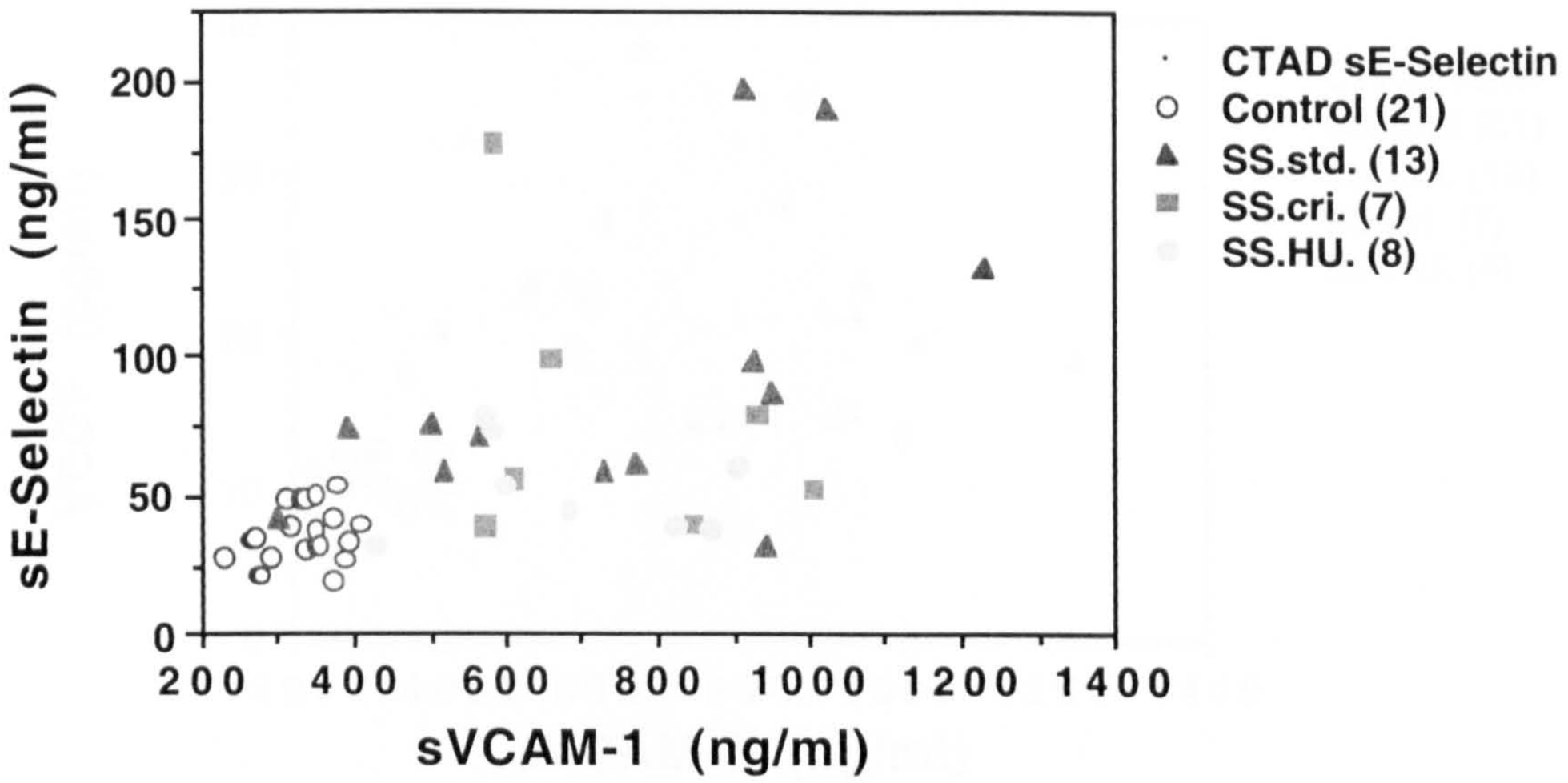


Figure 5.8.2 Dot-plot relationship between sE-Selectin and sVCAM-1

There was a weak correlation between sE-Selectin and sVCAM-1 in CTAD plasma. The correlation coefficients (r) were 0.56 in all subjects together, 0.29 in control group, 0.56 in SS.std. group, 0.34 in SS.cri. group and 0.13 in SS.HU. group respectively.

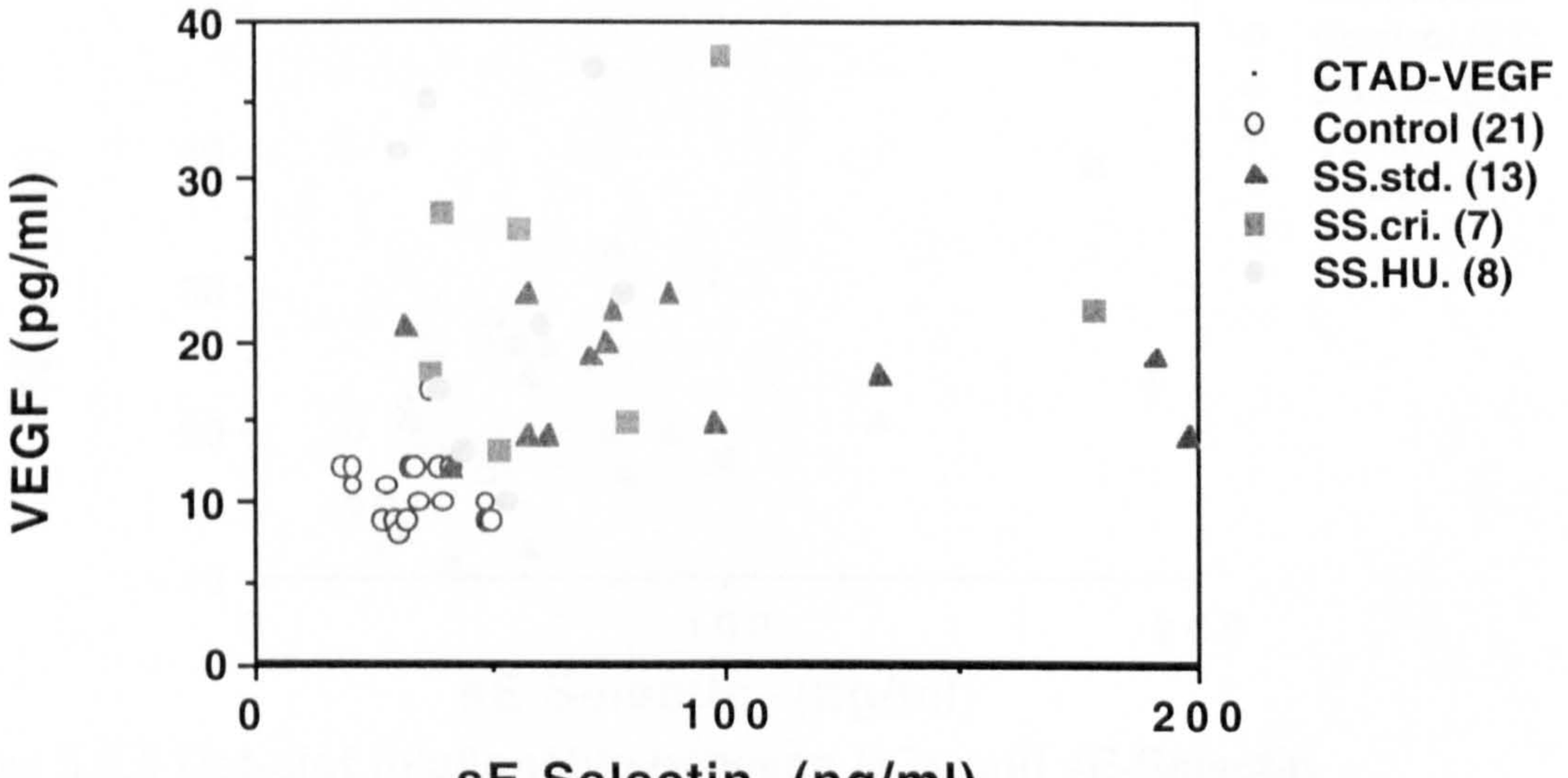


Figure 5.8.3 Dot-plot relationship between VEGF and sE-Selectin

There is no correlation between VEGF and sE-Selectin in CTAD plasma. The correlation coefficients (r) were 0.27 in all subjects together, 0.18 in control group, 0.11 in SS.std. group, 0.02 in SS.cri. group and 0.03 in SS.HU. group respectively.

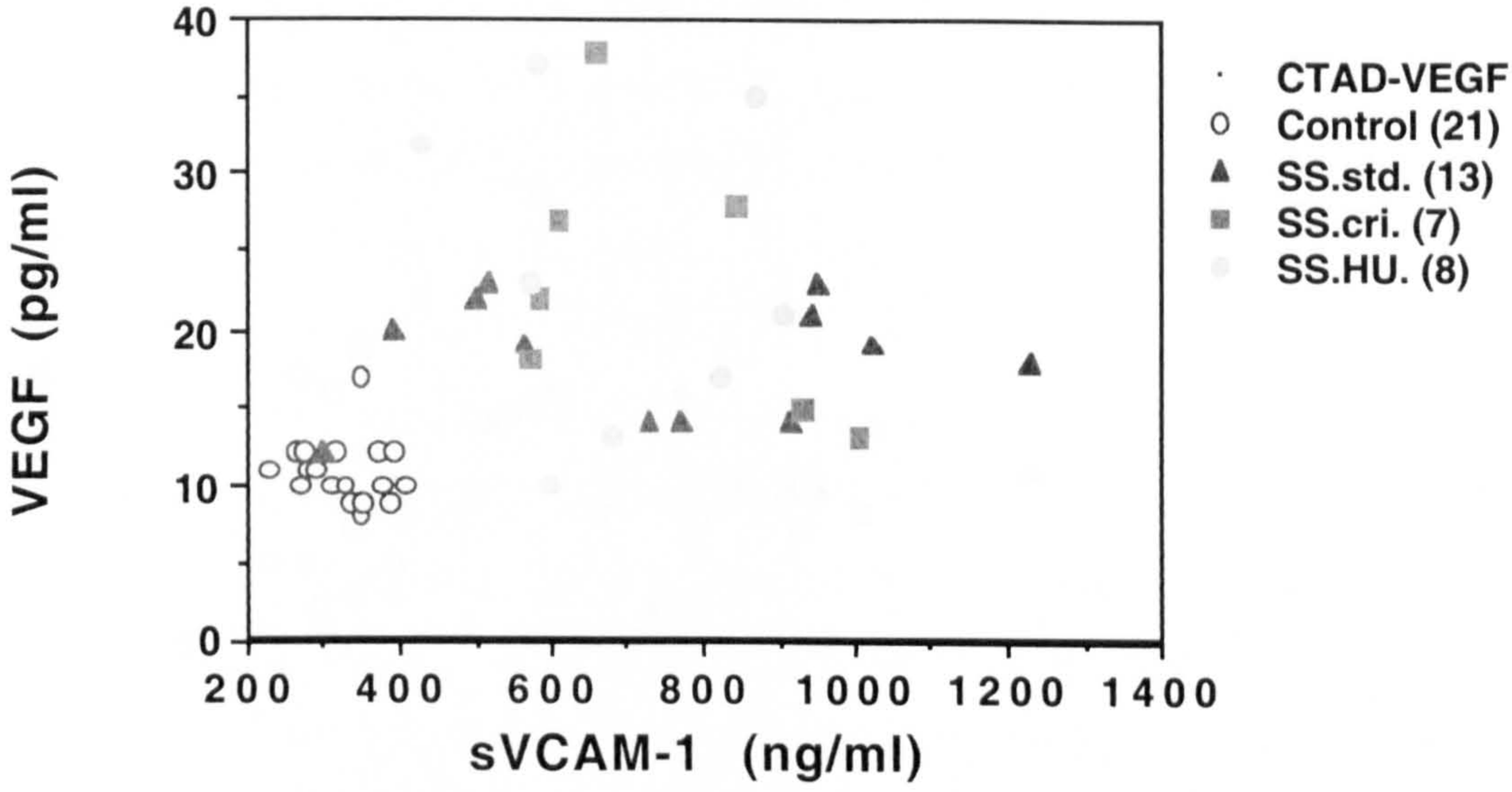


Figure 5.8.4 Dot-plot relationship between VEGF and sVCAM-1

There was no correlation between VEGF and sVCAM-1 in CTAD plasma. The correlation coefficients (r) were 0.42 in all subjects together, 0.11 in control group, 0.03 in SS.std. group, 0.47 in SS.cri. group and 0.13 in SS.HU. group respectively.

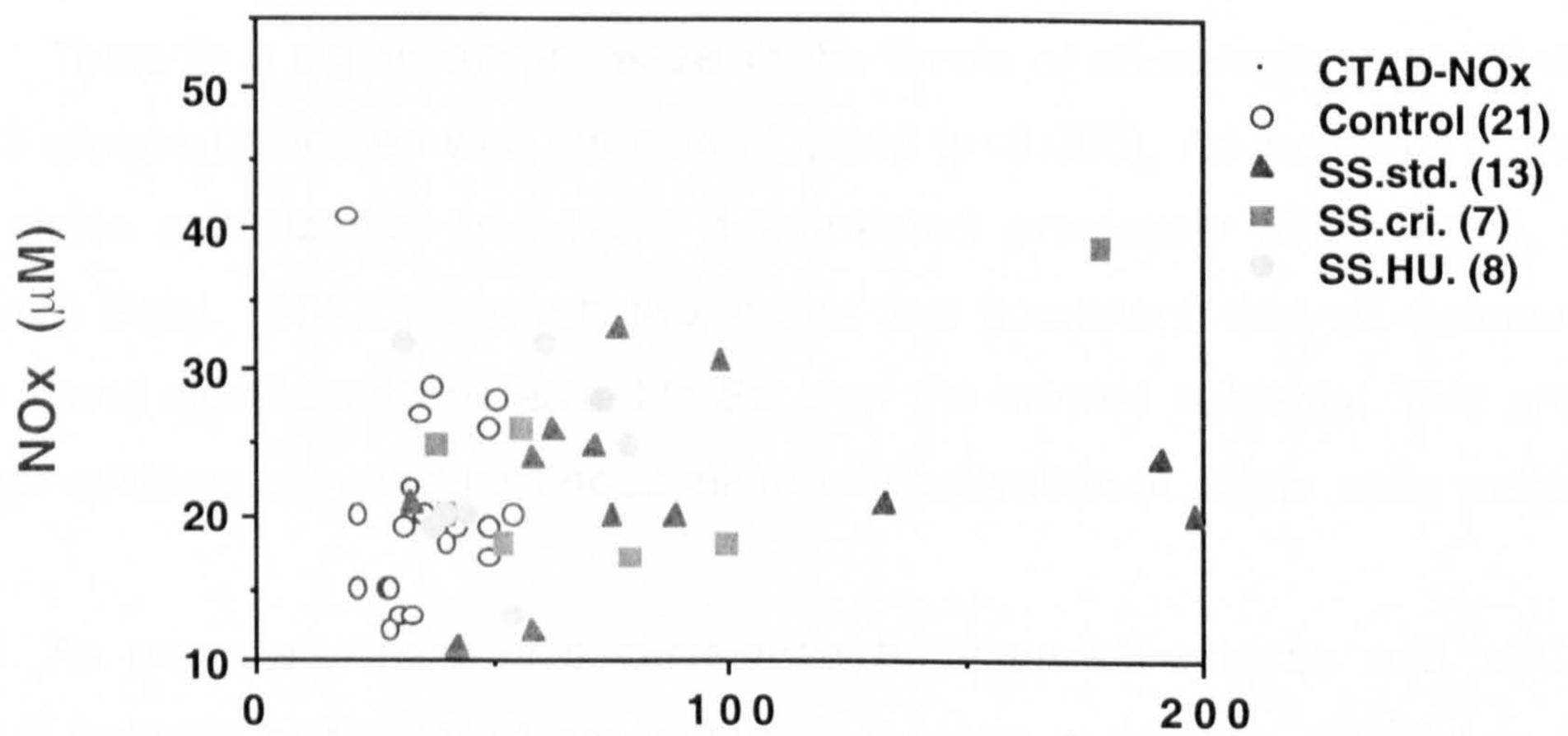


Figure 5.8.5 Dot-plot relationship between NOx and sE-Selectin

There was no correlation between NOx and sE-Selectin in CTAD plasma, except a weak correlation in SS.cri. The correlation coefficients (r) were 0.25 in all subjects together, 0.03 in control group, 0.15 in SS.std. group, 0.69 in SS.cri. group and 0.18 in SS.HU. group respectively.

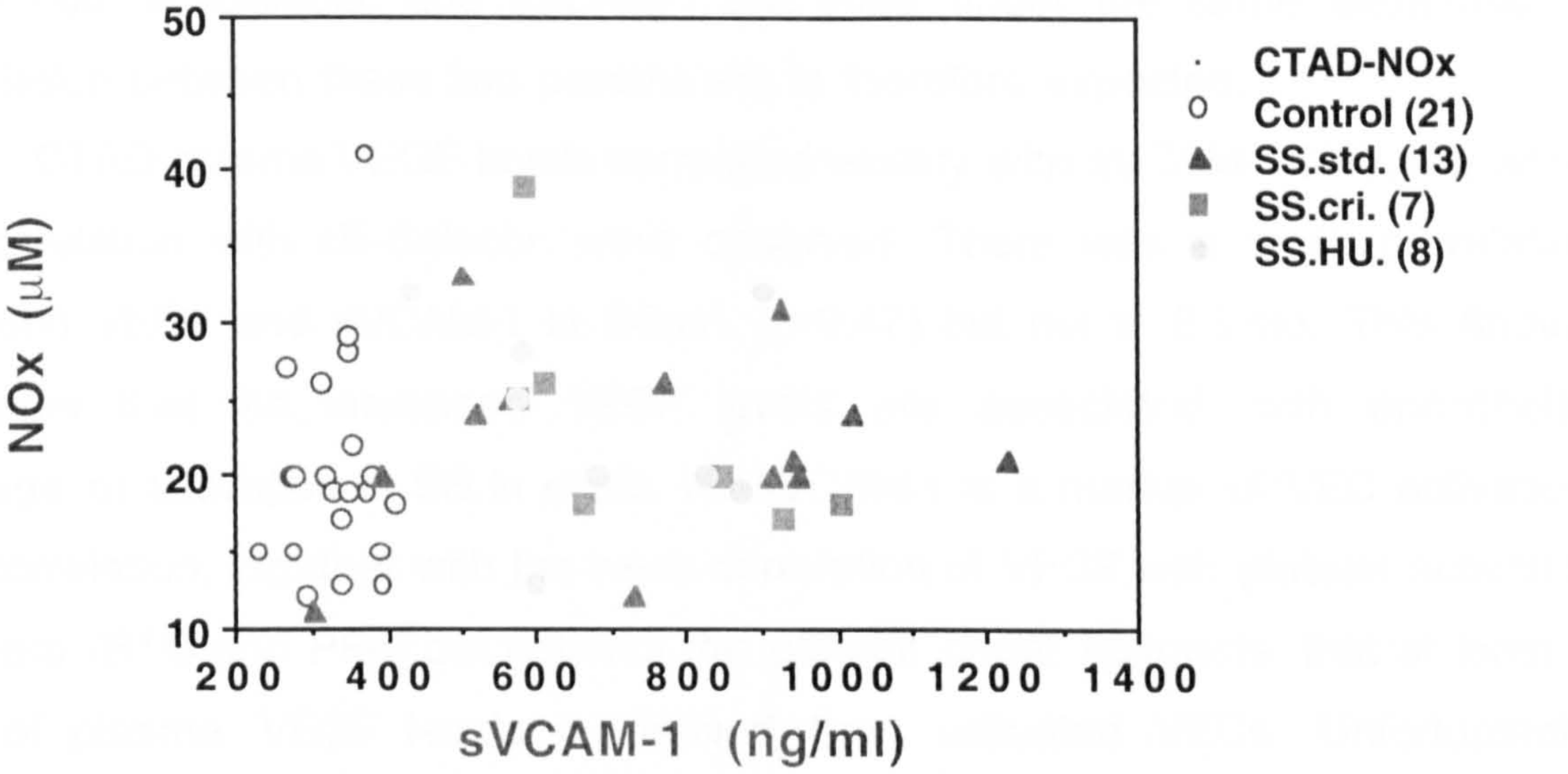


Figure 5.8.6 Dot-plot relationship between NOx and sVCAM-1

There was no correlation between NOx and sVCAM-1 in CTAD plasma, except a weak correlation in SS.cri. The correlation coefficients (r) were 0.01 in all subjects together, 0.12 in control group, 0.14 in SS.std. group, 0.67 in SS.cri. group and 0.17 in SS.HU. group respectively.

Discussion

There is a significant increase in the levels of sE-selectin and VCAM-1 in all SS groups compared with the control group ($p<0.005$). An increase in sVCAM-1 in sickle cell disease has been documented previously (Duits *et al.*, 1996; Stuart & Setty, 1999). However, this is the first document that sE-Selectin has been found significantly increased in SS over the normal subjects. This provides a clear evidence of vascular endothelium cell activation in sickle cells patients *in vivo*.

As expected, there is a correlation between sE-selectin and sVCAM-1. Both E-Selectin and VCAM-1 are cellular adhesion molecules (CAMs) found on vascular endothelial cells (VECs). CAMs also act as receptors for other adhesion molecules on the other cells. When VECs are activated, CAMs are upregulated and bound to their ligands on granulocytes, monocytes, lymphocytes and platelets. After activation, these CAMs are shed off to become the soluble forms (sE-Selectin and sVCAM-1) which neutralise their respective ligands on the other

cells. This is a negative feed back loop, which may prevent or delay adhesion. Since both sE-Selectin and sVCAM-1 are shed under the same situations, a correlation between these two parameters is therefore expected.

CTAD plasma VEGF levels correlated weakly with sVCAM-1 (r=0.42), while no correlation with sE-Selectin were observed. There was a weak correlation between VEGF and sVCAM-1 in SS.cri. (r=0.47) but not in SS.std. This finding indicates that the increased VEGF levels are associated with endothelial damage or activation in SS in crisis. As sVCAM-1 is a marker of VEC activation, this correlation, together with the weak correlation of VEGF with platelet activation markers (BTG and PF4) but not with the platelet count suggests that at least a part of plasma VEGF levels is derived from activated VECs. Unfortunately, sVCAM-1 is not a specific marker of VEC activation, for example dendritic cells may also shed VCAM-1. Another more specific VEC activation marker, sE-Selectin, does not correlate with VEGF either in steady state or in crisis. However, the mean levels of sE-Selectin in all 3 SS groups were significantly higher than the control group, showing evidence of VEC activation in sickle cell disorders.

NOx did not correlate with endothelial activation markers in SS as a whole. However, there were correlation of NOx with both sE-Selectin (r=0.69) and sVCAM-1 (r=0.66) in SS.cri. but this correlation is probably dependent on a single point, which is high for both values, otherwise there is no correlation. Interpretation should be made with caution in this small number of samples. There is also no correlation between NOx with either sVCAM-1 or with sE-Selectin in control, SS.std. and SS.HU. groups

Taken together, the findings in this section provide clear evidence of vascular endothelial cell activation in sickle cell disorders. In overall there is no clear correlation between endothelial activation markers and NOx but weak correlation between VEGF and sVCAM-1 both in all subjects together and in crisis SS were found.

5.9. RELATIONSHIP OF PLASMA VEGF AND NOx TO ANAEMIC MARKERS

Rationale

Erythropoietin (Epo), a glycoprotein of 30.4 kDa produced by kidney cells, is the principal hormone regulating red cell production (erythropoiesis). Production of Epo is regulated by changes in oxygen availability to the kidney cells. Epo-producing cells are the fibroblast-like type I interstitial cells (Maxwell *et al.*, 1993) or peritubular cells (Lacombe *et al.*, 1988).

VEGF and Epo are both activated by a similar hypoxic mechanism, Epo and Hb were examined to see if there was any relationship with VEGF and NOx. It was important to determine whether plasma VEGF levels responded to generalised hypoxia, resulting from low Hb, or whether the local ischaemia of vaso-occlusion was more important. Haemoglobin (Hb) and erythropoietin (Epo) were therefore compared with VEGF and NOx of normal control subjects, production defect anaemic patients, and sickle cell disorders.

5.9.1. Comparison of EDTA plasma VEGF, NOx, Epo and blood Hb levels

Blood samples

EDTA plasma samples were taken from 20 control subjects, 8 sickle cell patients in steady state (SS.std.), 8 sickle cell patients in crisis (SS.cri.), 7 sickle cell patients undergoing hydroxyurea treatment and 12 production anaemic patients (iron, vitamin B12 or folate deficiency patients, Table 5.9.1). Epo, VEGF and NOx levels were determined using commercial ELISA test kits (section 2.5.3) and blood Hb levels were obtained from routine FBC data. These parameters were performed on EDTA plasma prior to the finding that CTAD was more suitable for VEGF measurement. A subsequent study (section 5.9.2) examines the relationship between VEGF, NOx and Epo, using CTAD plasma.

Table 5.9.1 Diagnosis and clinical data of productional anaemic patients

No.	Age	Sex	Diagnosis	Hb	B12	Folate	SI	IBC	Ferritin
1.	76	M	Fe def.	6.9	816	655	3.2	52	-
2.	83	M	B12 def.	8.1	112	-	-	-	-
3.	86	F	Fe & Fol def.	7.3	285	204	1.7	65	64
4.	93	F	Fol def.	7.3	-	282	11.3	34	270
5.	88	M	Fe def.	10.7	-	-	6.1	63	-
6.	43	F	Fe def.	10.4	-	-	4.2	66	34
7.	55	F	Fe & Fol def.	6.6	502	31	5.6	26	-
8.	27	F	Fe & Fol def.	5.8	273	405	1.8	72	4
9.	63	F	Fe def.	7.6	-	-	2.0	65	6
10.	82	M	Fe def.	9.8	603	784	2.9	62	-
11.	49	F	Fe def.	7.4	-	-	2.7	61	5
12.	30	F	Fe def.	10.0	-	-	3.8	80	4

B12 = vitamin B12 (200-1000 pg/ml), Folate = red cell folate (550-2200 nM), SI = serum iron (7-45 uM), IBC = iron binding capacity (male 20-50, female 15-50 %), Feritin = serum feritin (male 30-284, female 6-186 μg/ml), Age = years, M = male, F = female, Fe = iron, Fol = folate, def = deficiency, ND = No data

Results

Blood Hb levels in normal control subjects (n=20), SS.std. (n=8), SS.cri. (n=8), SS.HU. (n=7) and production anaemic patients (n=12) were 14.2±0.3, 8.4±0.3, 7.9±0.6, 8.7±0.5 and 8.2±0.5 g/dl, respectively. Levels of EDTA plasma Epo in controls, SS.std., SS.cri., SS.HU. and anaemic patients were 3±0.5, 53±8.0, 61±8.9, 47±6.1 and 46±9.7 mIU/ml, respectively. Levels of EDTA plasma VEGF in controls, SS.std., SS.cri., SS.HU. and anaemic patients were 22±2.6, 115±51.6, 73±9.9, 170±53.3 and 46±9.7 pg/ml respectively. Levels of EDTA plasma NOx in controls, SS.std., SS.cri., SS.HU. and anaemic patients were 27±3.0, 25±2.4, 25±2.1, 28±4.9 and 32±7.2 μM, respectively. The Epo levels in all 4 groups of patients were significantly higher than in the control group ($p<0.001$), but no significant difference between SS.std., SS.cri. and SS.HU. groups were observed. The Hb level was significantly lower in all 4 patient groups than in the control group ($p<0.001$), but no significant different between SS.std., SS.cri. and SS.HU. was observed (Figure 5.9.1.1). The dot-plot relationship between Hb and Epo is shown in Figure 5.9.1.2, between VEGF and Hb in Figure 5.9.1.3, between

VEGF and Epo in Figure 5.9.1.4, between NOx and Hb in Figure 5.9.1.5 and between NOx and Epo in Figure 5.9.1.6 respectively.

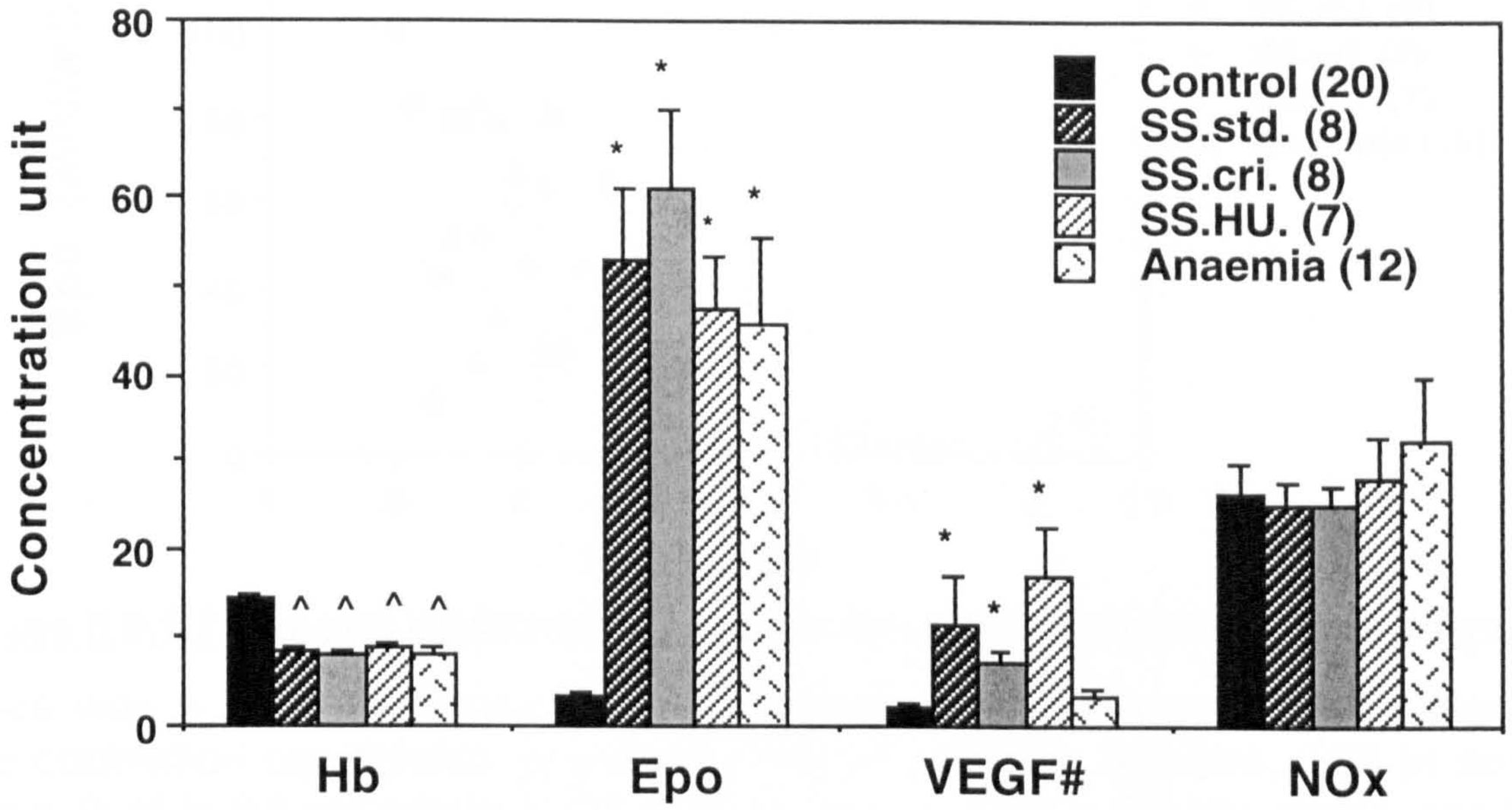


Figure 5.9.1.1 Levels of blood Hb, EDTA plasma Epo, VEGF and NOx

Comparison of blood Hb (g/dl) and EDTA plasma Epo (mIU/ml), VEGF (#x10pg/ml) and NOx (μM) levels in normal control subjects (Control, n=20), sickle cell patients in steady state (SS.std., n=8), sickle cell patients in crisis (SS.cri., n=8), sickle cell patients undergoing hydroxyurea treatment (SS.HU., n=7) and production anaemic patients (Anaemia, n=12) were shown. *Indicates significantly higher than control group ($p<0.001$). ^Indicates significantly lower than control group ($p<0.05$). There was no significant difference among SS.std., SS.cri. and SS.HU. groups in any parameter.

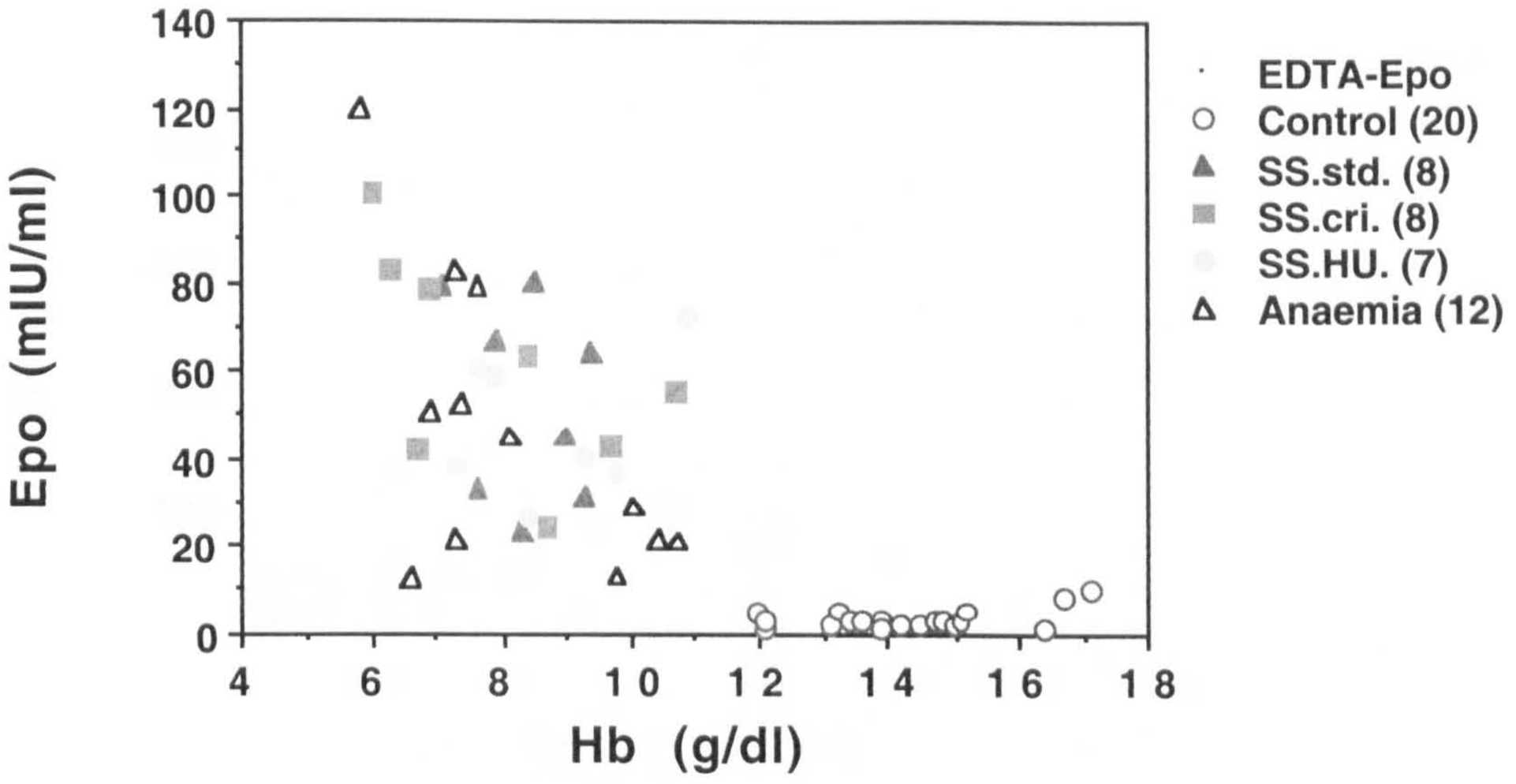


Figure 5.9.1.2 Dot-plot relationship between blood Hb and EDTA plasma Epo

There was a strong inverse correlation between Hb and Epo in EDTA plasma. The correlation coefficients (r) were 0.77 in all subjects together, 0.50 in control group, 0.25 in SS.std. group, 0.60 in SS.cri. group, 0.26 in SS.HU. group and 0.61 in anaemic group respectively.

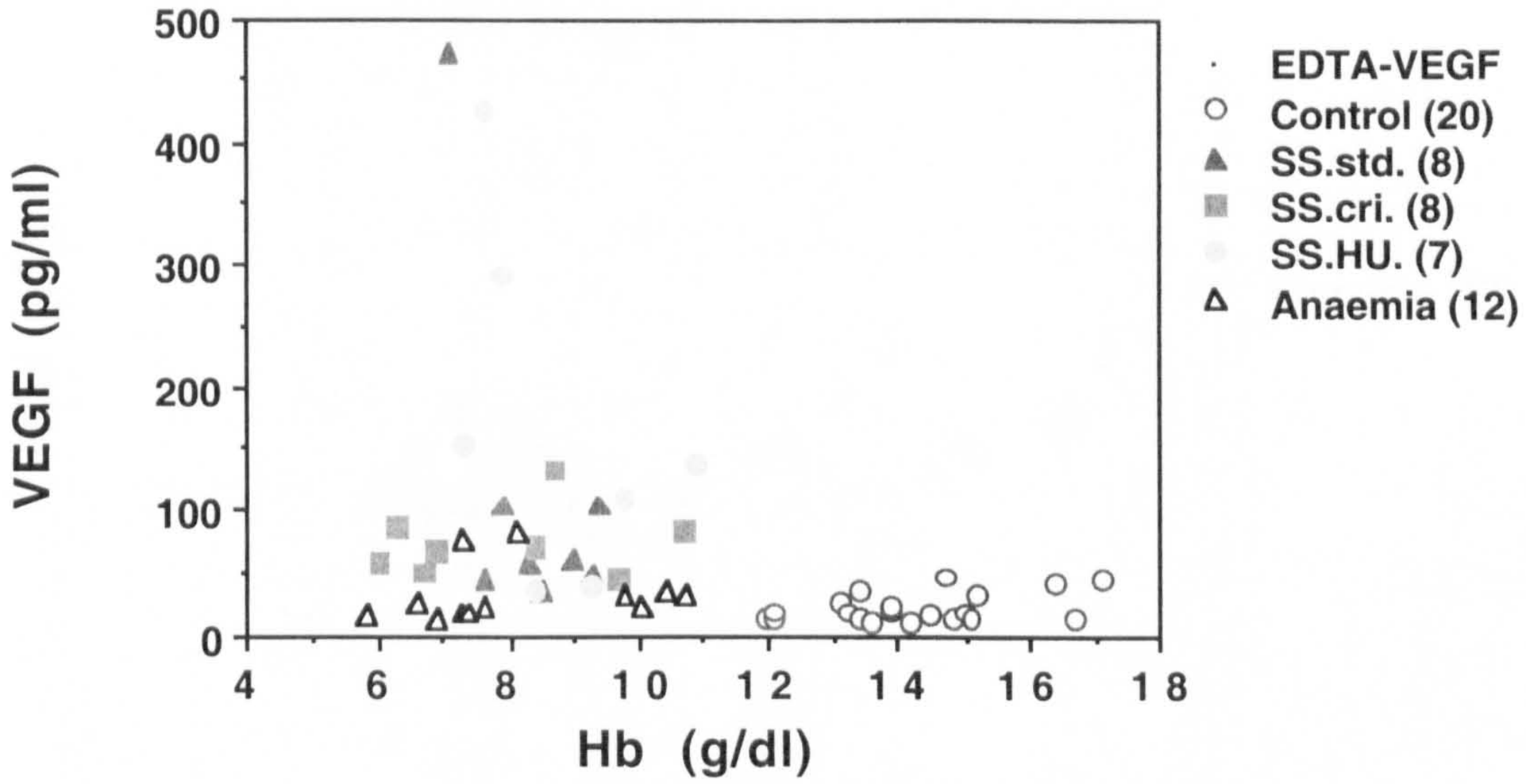


Figure 5.9.1.3 Dot-plot relationship between EDTA plasma VEGF and blood Hb

There was no correlation between VEGF and Hb in EDTA plasma, except a weak correlation in SS.std. The correlation coefficients (r) were 0.35 in all subjects together, 0.39 in control group, 0.60 in SS.std. group, 0.18 in SS.cri. group, 0.48 in SS.HU. group and 0.14 in anaemic group respectively.

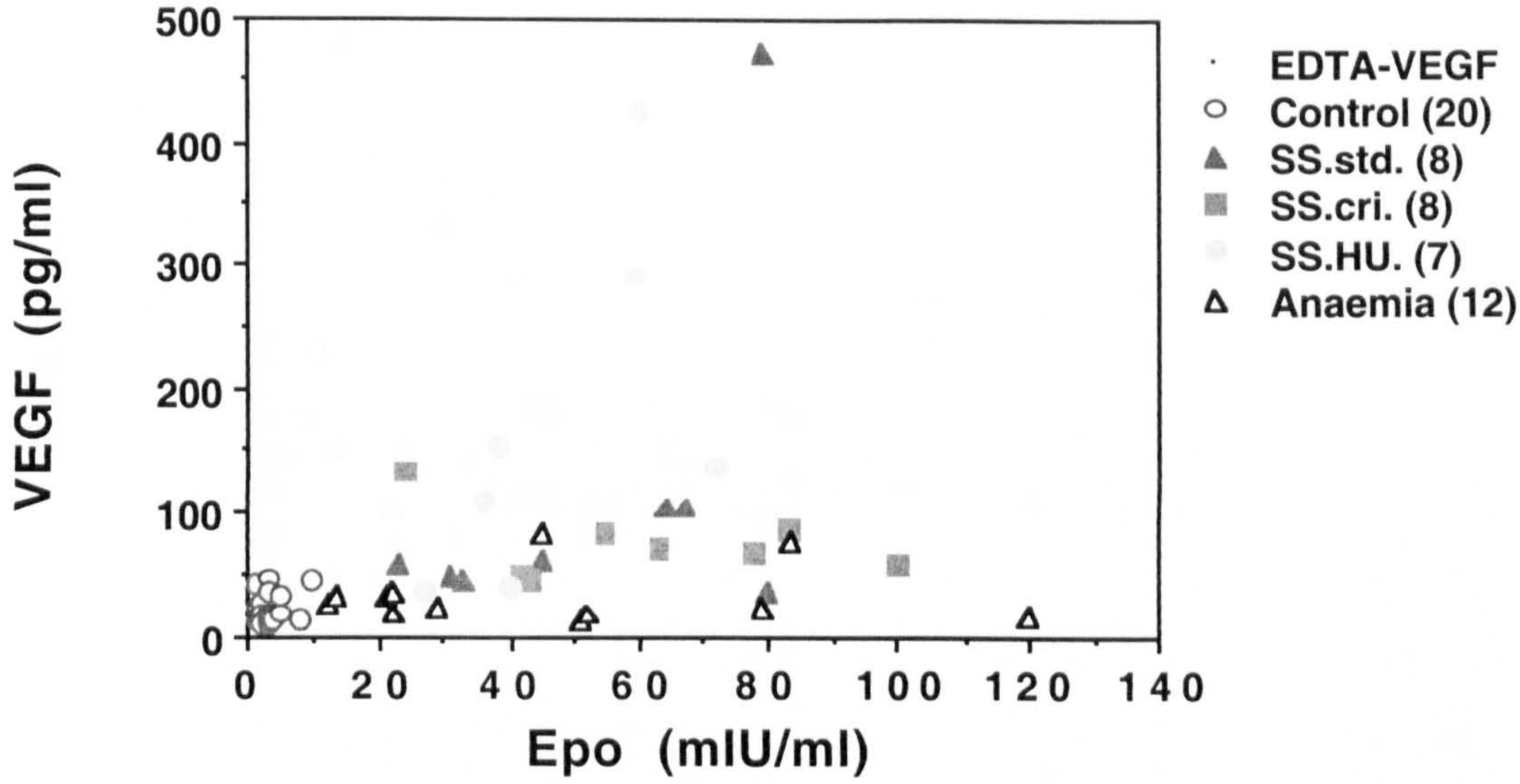


Figure 5.9.1.4 Dot-plot relationship between EDTA plasma VEGF and Epo

There was no correlation between VEGF and Epo in EDTA plasma, except a weak correlation in SS.std. and SS.HU. The correlation coefficients (r) were 0.40 in all subjects together, 0.20 in control group, 0.52 in SS.std. group, 0.33 in SS.cri. group, 0.61 in SS.HU. group and 0.06 in anaemic group respectively.

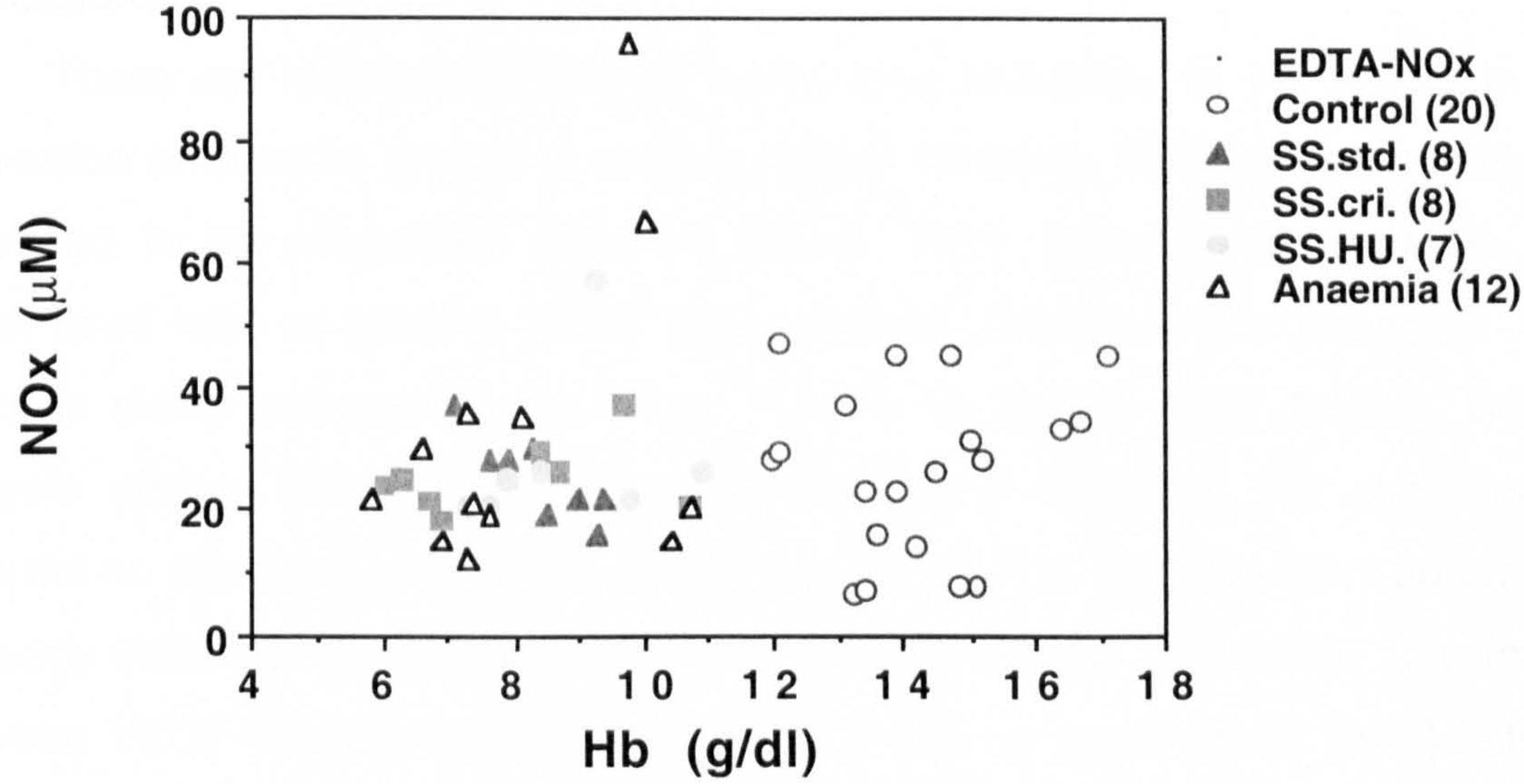


Figure 5.9.1.5 Dot-plot relationship between EDTA plasma NOx and blood Hb

There was no correlation between NOx and Epo in EDTA plasma. The correlation coefficients (r) were 0.06 in all subjects together, 0.13 in control group, 0.46 in SS.std. group, 0.34 in SS.cri. group, 0.29 in SS.HU. group and 0.40 in anaemic group respectively.

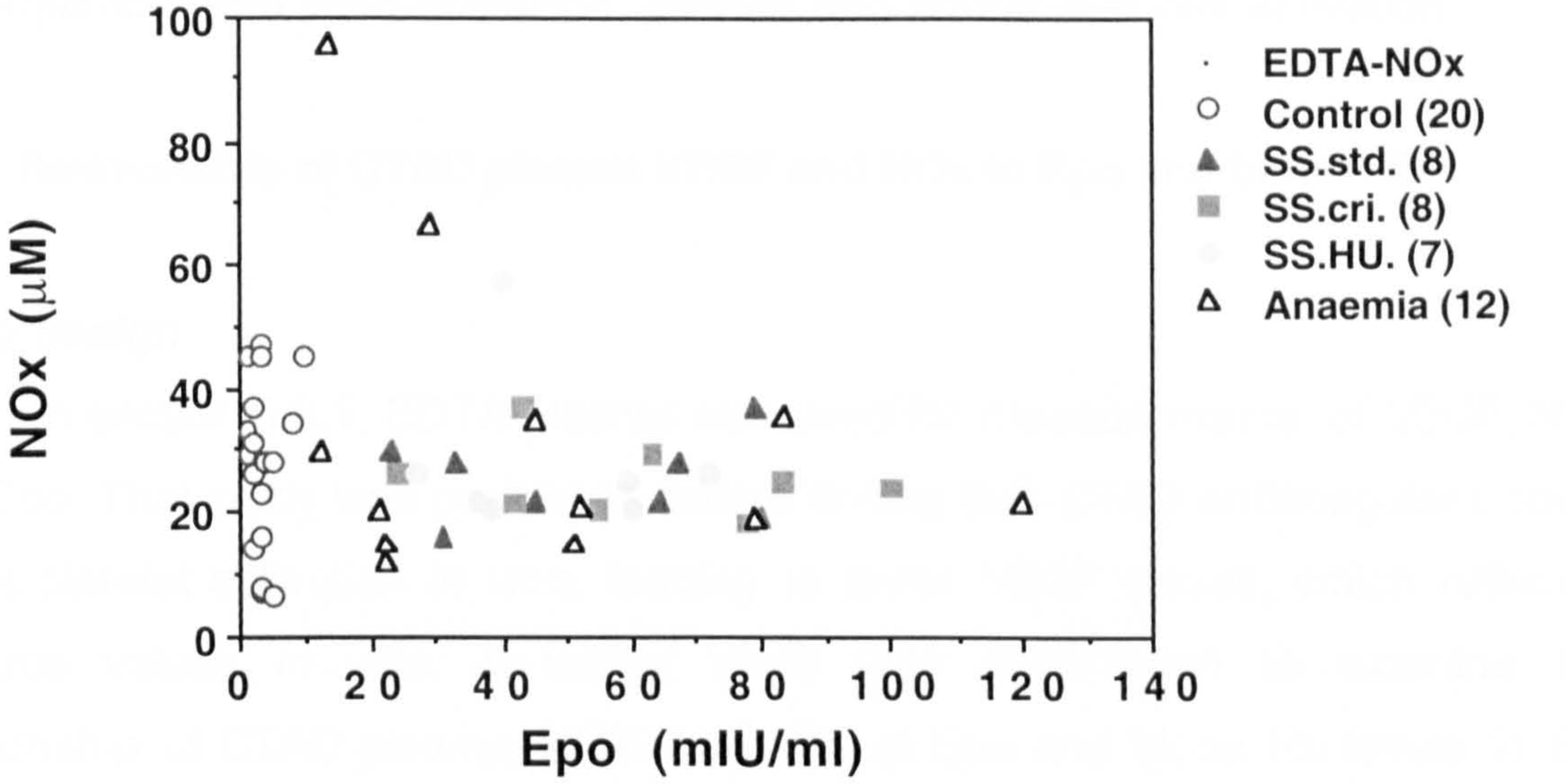


Figure 5.9.1.6 Dot-plot relationship between EDTA plasma NOx and Epo

There was no correlation between NOx and Epo in EDTA plasma. The correlation coefficients (r) were 0.10 in all subjects together, 0.13 in control group, 0.16 in SS.std. group, 0.28 in SS.cri. group, 0.17 in SS.HU. group and 0.29 in anaemic group respectively.

Discussion

There are increments of Epo levels and reduction in Hb levels in both production amaemias and all 3 sickle groups. However VEGF is not significantly increased in the production anaemia group. Thus, generalised anaemia does not in itself lead to plasma VEGF upregulation. Therefore the raised in VEGF levels in sickle cell groups are most likely to be due to other factors. Dot plot analysis shows the expected inverse relationship between Hb and Epo but confirms no relationship between VEGF and Hb or Epo in production anaemia or in steady state SS. In HU-treated SS, there appears to be a possible relationship between VEGF with Hb and with Epo but the numbers are small (n=7). Plasma NOx values are unaltered in anaemic or sickle patients and do not correlate with either Hb or Epo levels.

The findings in this section therefore suggest that the raised VEGF values seen in SS are independent of generalised hypoxia or anaemia and are likely to

result from other causes. The most likely causes are expected to be local hypoxia consequence from vaso-occlusion, platelet and endothelial cell activation.

5.9.2. Relationship of CTAD plasma VEGF and NOx to Epo and blood Hb

Study design

In section 5.9.1, EDTA plasma was used for measurements of VEGF, NOx and Epo. That study was performed before finding that CTAD anticoagulant could inhibit platelet activation *in vitro,* leading to lower VEGF values, which reflected the true values *in vivo*. A further study was undertaken to examine the relationship of CTAD plasma VEGF and NOx to Epo and blood Hb levels in SS. Blood samples were obtained from 21 control subjects (Control), 13 sickle cell patients in steady state (SS.std.), 7 sickle cell patients in crisis (SS.cri.) and 8 sickle cell patients undergoing hydroxyurea treatment (SS.HU.) and were assayed for these parameters using commercial test kits (section 2.5.3). Hb levels of these samples were obtained from routine FBC data.

Results

Levels of blood Hb (g/dl) in the controls (n=21), SS.std. (n=13), SS.cri. (n=7) and SS.HU. (n=8) were 13.8±0.3, 8.6±0.5, 7.4±0.5 and 8.5±0.4 g/dl respectively, with significantly lower levels in all 3 SS groups compared to in control group ($p<0.001$), but no significant difference was observed between SS.std., SS.cri. and SS.HU. Levels of CTAD plasma Epo (mIU/ml) in the controls, SS.std., SS.cri. and SS.HU. were 4±0.4, 62.9±10.7, 59±5.5 and 80±14.9 respectively, with significantly higher values in all 3 SS groups than in the control group ($p<0.0001$), but no significant difference was observed between SS.std., SS.cri. and SS.HU. groups (Figure 5.9.2.1). The dot-plot relationship between Hb and Epo is shown in Figure 5.9.2.2, between VEGF and Hb is shown in Figure 5.9.2.3, between VEGF and Epo in Figure 5.9.2.4, between NOx and Hb in Figure 5.9.2.5 and between NOx and Epo in Figure 5.9.2.6 respectively.

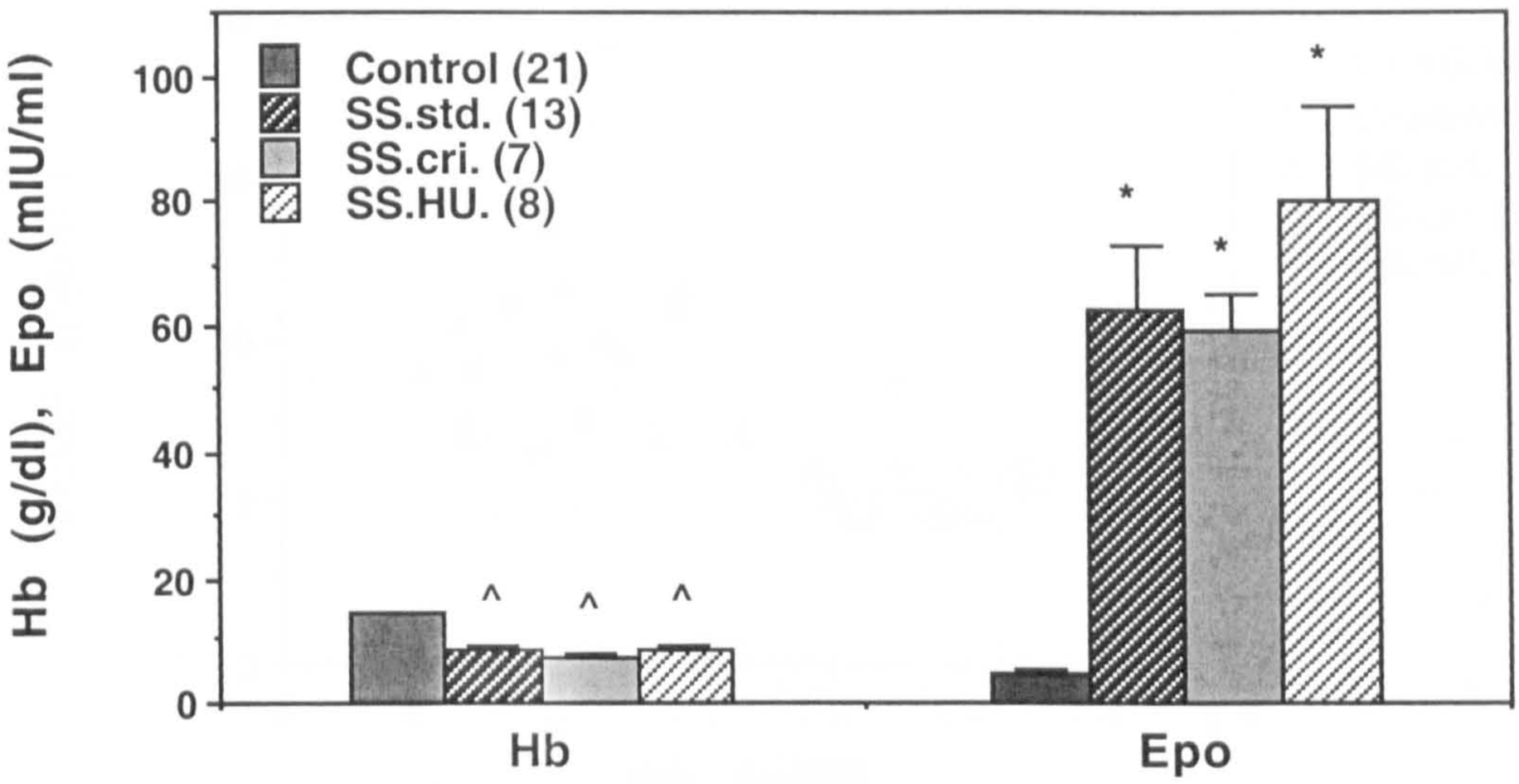


Figure 5.9.2.1 Comparison of levels of blood Hb and CTAD plasma Epo

CTAD plasma from 21 normal control subjects, 13 sickle cell patients in steady state (SS.std.), 7 sickle cell patients in crisis (SS.cri.) and 8 sickle cell patients undergoing hydroxyurea treatment (SS.HU.) was assayed for Epo (mIU/ml) and Hb concentrations were obtained from FBC data. *Indicates significantly higher than control group ($p<0.001$). ^Indicates significantly lower than control group ($p<0.05$).

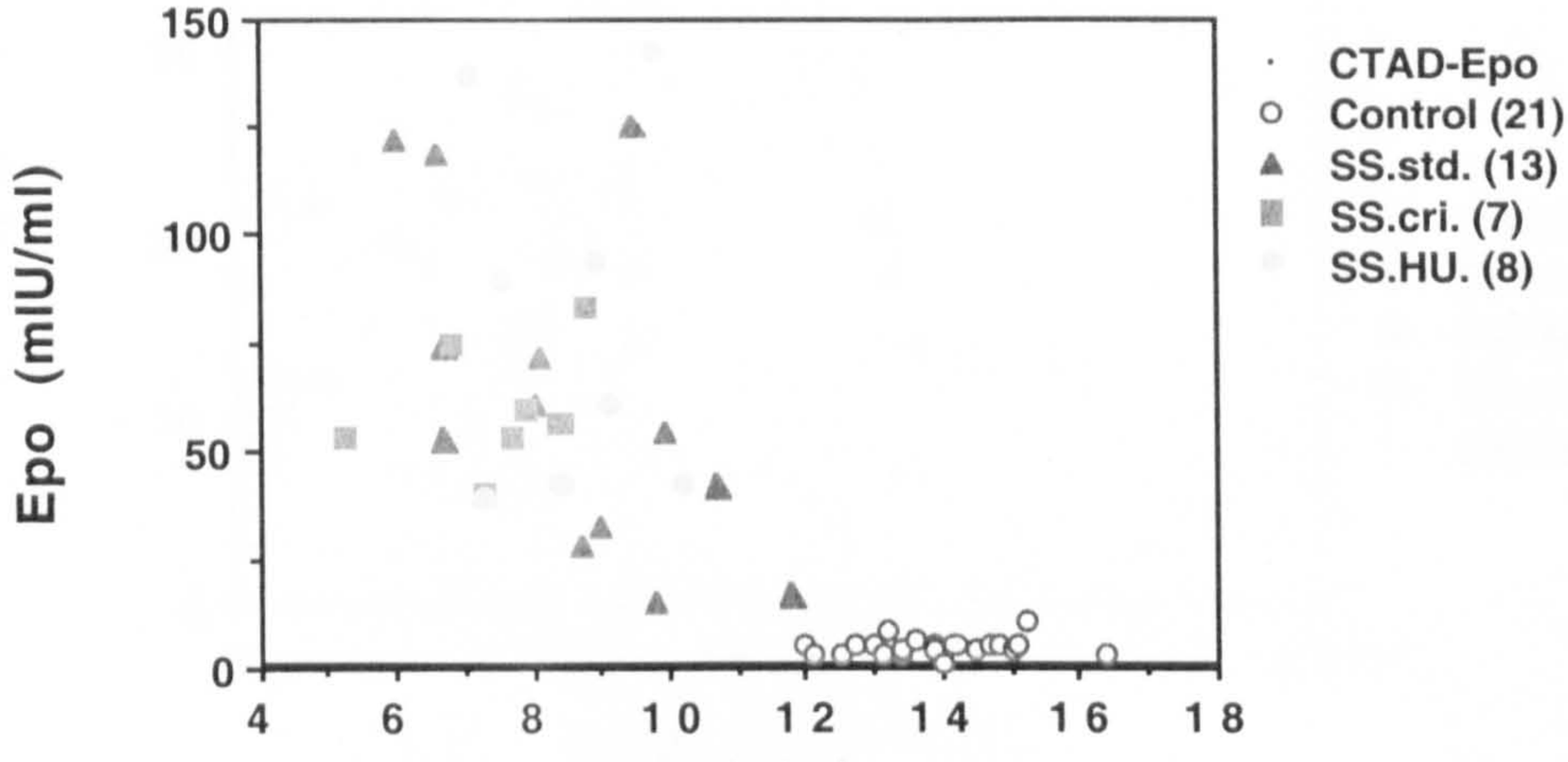


Figure 5.9.2.2 Dot-plot relationship between blood Hb and CTAD plasma Epo

There was a strong inverse correlation between Hb and CTAD plasma Epo in overall and in SS.std. group. The correlation coefficients (r) were 0.75 in all subjects together, 0.20 in control group, 0.60 in SS.std. group, 0.32 in SS.cri. group and 0.08 in SS.HU. group respectively.

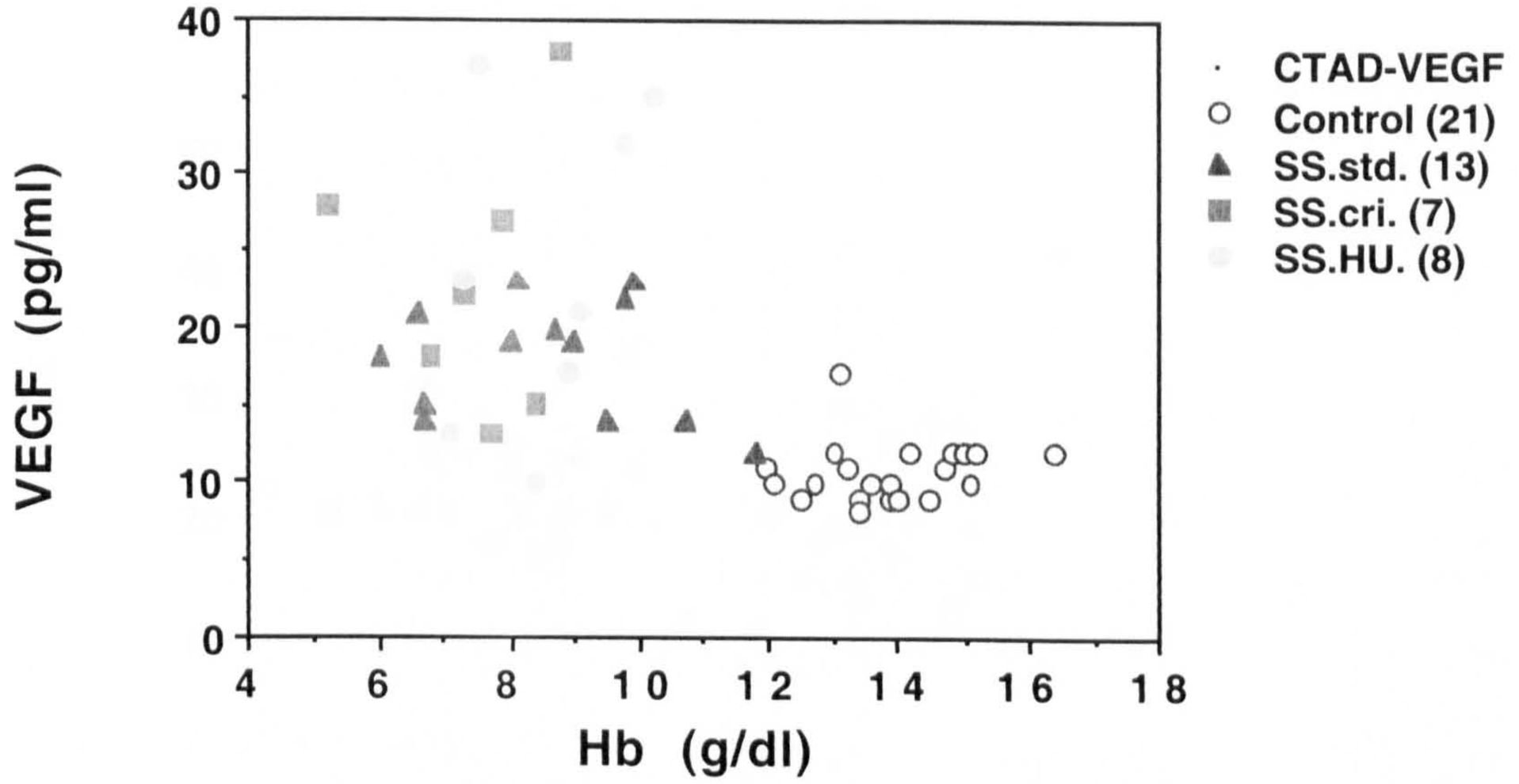


Figure 5.9.2.3 Dot-plot relationship between blood Hb and CTAD plasma VEGF

There was a weak correlation between Hb and CTAD plasma VEGF in over all group. The correlation coefficients (r) were 0.58 in all subjects together, 0.14 in control group, 0.19 in SS.std. group, 0.06 in SS.cri. group and 0.37 in SS.HU. group respectively.

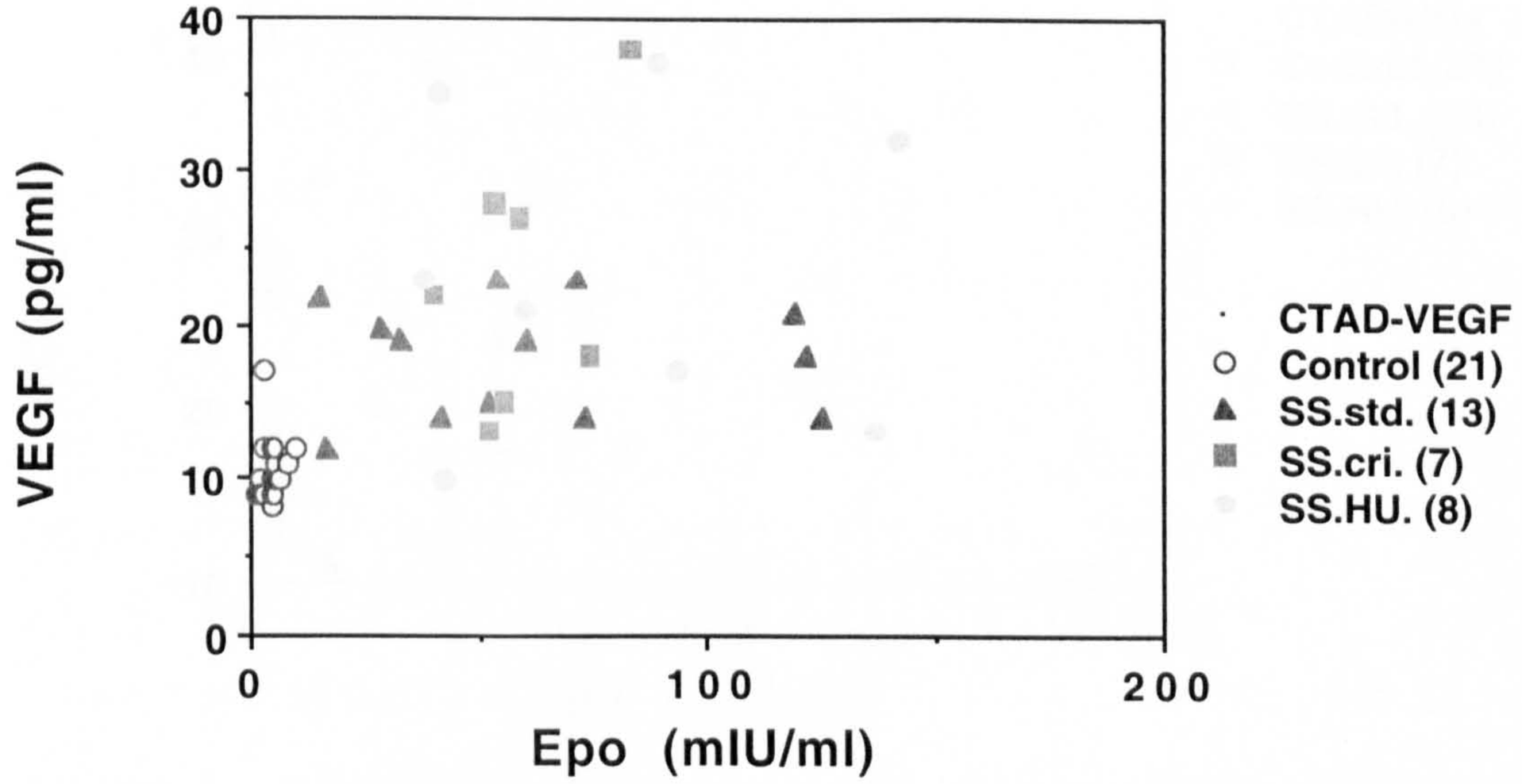


Figure 5.9.2.4 Dot-plot relationship between CTAD plasma VEGF and Epo

There was a weak correlation between Hb and CTAD plasma Epo in over all group. The correlation coefficients (r) were 0.55 in all subjects together, 0.17 in control group, 0.00 in SS.std. group, 0.49 in SS.cri. group and 0.03 in SS.HU. group respectively.

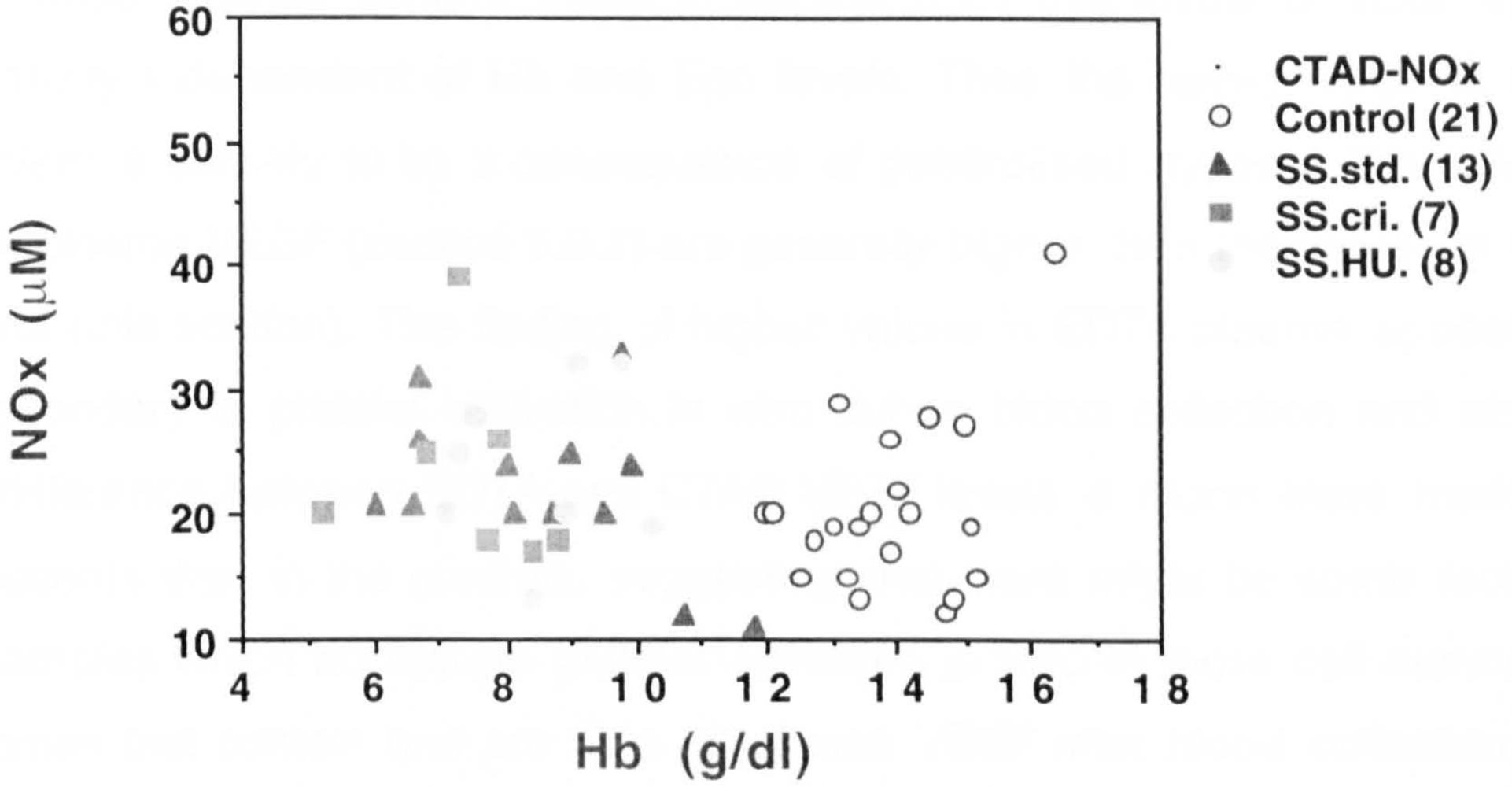


Figure 5.9.2.5 Dot-plot relationship between blood Hb and CTAD plasma NOx

There was no correlation between Hb and CTAD plasma NOx. The correlation coefficients (r) were 0.18 in all subjects together, 0.37 in control group, 0.46 in SS.std. group, 0.17 in SS.cri. group and 0.12 in SS.HU. group respectively.

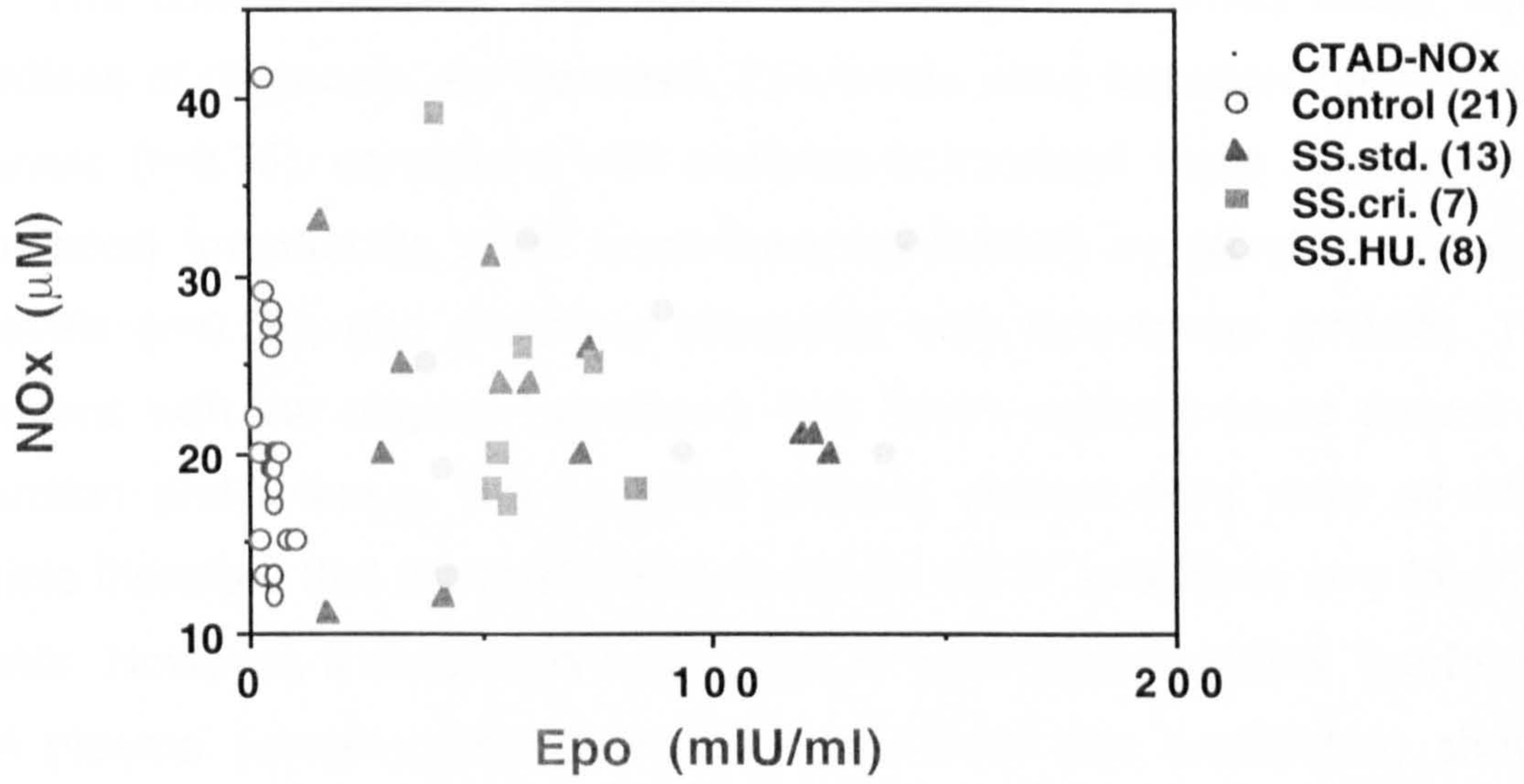


Figure 5.9.2.6 Dot-plot relationship between CTAD plasma NOx and Epo

There was no correlation between CTAD plasma NOx and Epo. The correlation coefficients (r) were 0.16 in all subjects together, 0.35 in control group, 0.06 in SS.std. group, 0.47 in SS.cri. group and 0.33 in SS.HU. group respectively.

Discussion

These findings confirm those in section 5.9.1 that levels of VEGF appear essentially independent of Hb and Epo levels. Thus the raised VEGF in sickle disorders is unlikely to be a consequence of generalised hypoxia. The values of EDTA plasma VEGF (section 5.9.2) are generally higher than the values in CTAD plasma (this section). The finding of higher values in EDTA plasma appeared to be secondary to platelet activation *in vitro* during blood collection and storage. The difference between EDTA and CTAD VEGF levels is much more marked in SS patients than in the controls, suggesting that there might be some factors in SS samples which accelerate platelet activation *in vitro* or more cell numbers or cell types that contain and are able to release VEGF after blood collection. With respect to the relationship between Hb and Epo with VEGF, the conclusions from the findings in EDTA and CTAD plasma studies are essentially the same. The firm evidence found is that generalised hypoxia does not account for increased plasma VEGF levels.

The correlation was determined in a total of 49 CTAD blood samples regardless of diagnosis. As expected, Epo levels were negatively correlated with Hb levels (r=0.75), consistent with anaemia-associated renal hypoxia inducing Epo release. Importantly, VEGF levels were significantly negatively correlated with Hb levels (r=0.58), and positively correlated with Epo levels (r=0.55). This is consistent with the original hypothesis that tissue hypoxia could induce VEGF production and release. The anaemic patients studied here were all SS. It is possible therefore that the relationship between VEGF and Hb is only found in SS patients. However, it should be noted that, in preliminary studies (performed in EDTA plasma samples before knowing that CTAD was preferable) showed a weak negative correlation between VEGF and Hb and a weaker positive correlation between VEGF and Epo (r=0.35 and 0.40 respectively).

The levels of Epo in all 3 groups of SS were higher but the Hb levels were lower than in the control group. The high Epo levels result from the anaemic status in these patients. In addition, the CTAD plasma VEGF level was significantly correlated with Epo and with Hb, with the stronger correlation than in

EDTA plasma. This indicates that VEGF shares the same anaemic or low O_2 responsive process with Epo and Hb. The relationship appeared stronger in CTAD than in EDTA plasma because the interference of VEGF release from platelet activation *in vitro* was prevented. There was no correlation between NOx levels in CTAD plasma with either Epo or Hb.

5.10. CONCLUSIONS

The principle finding in this chapter is that plasma VEGF is raised in sickle cell disorders, a finding not previously reported when this work was started. This finding has since been confirmed by others (Solovey *et al.*, 1999).

The original hypothesis which triggered the work in this chapter was that as VEGF synthesis was controlled by a hypoxia sensitive element in vascular endothelial cells (VECs), microvascular occlusion and consequent tissue hypoxia in sickle cell disorders would lead to increased VEGF synthesis in VECs. The initial finding in this thesis that VEGF was raised in serum samples of SS patients could have been interpreted as consistent with this hypothesis. However, it soon became apparent that *in vitro* platelet activation was responsible for the majority of serum VEGF. This was not reported at the commencement of this work but has subsequently been confirmed by a variety of investigators in non-sickle patients. Interestingly, it appears that VEGF release *in vitro* is greater in SS patients than in control subjects, suggesting a greater tendency to platelet activation *in vitro* in sickle cell disorders. The reason for this has not been investigated in this thesis, but as platelet activation is a recognised feature of SS and sickle red cells or red cell spicules are known to interact with platelets, it is likely that this process occurs *in vitro* during collection of the serum samples. It is clear that CTAD, which inhibits *in vitro* platelet activation also inhibits VEGF release *in vitro*, but that even in CTAD plasma, VEGF is significantly greater in SS patients than in control subjects.

The question therefore arises as to the mechanism of raised VEGF levels and whether this results from hypoxia-mediated vaso-occlusion or from platelet or endothelial activation. The evidence presented in this thesis is inconclusive in this regard but offers some clues. It is clear that generalised hypoxia, such as occurs with anaemia, is not the cause of the raised VEGF. This is because VEGF is not raised in production anaemia, a new finding, and secondly because VEGF does not correlate with Epo or Hb in production anaemic or in SS patients. There is no obvious relationship between VEGF and markers of endothelial activation, sE-Selectin and sVCAM-1, although both are raised in SS patients relative to healthy control subjects. Raised sVCAM-1 has been reported previously in SS patients but not in sE-selectin. A lack of correlation between VEGF and these endothelial markers does not exclude an endothelial origin of VEGF but suggests that the factors determining plasma levels of VEGF and endothelial makers are not regulated in the same way.

The evidence with respect to platelet activation requires careful scrutiny. In CTAD plasma, VEGF levels in SS patients are approximately 2 fold higher than in the control group, whereas BTG and PF4, markers of platelet activation, are raised by approximately 6 and 4 fold in SS patients over the control subjects. Thus VEGF is raised in SS patients proportionally less than markers of platelet activation. However, production of VEGF is not restricted to platelets. There are many sources of VEGF, which may associated with raised plasma VEGF such as vascular endothelial cells, vascular muscle cells, leukocytes and platelets.

CHAPTER 6

GENERAL CONCLUSION AND FUTURE TRENDS

6.1. KEY THEMES OF THE THESIS

Although the basic pathophysiology of sickle cell anaemia (SS) is well known, the sources of variability in disease severity are incompletely understood. In this thesis a number of factors which have the potential to modify the severity of SS have been examined. The importance of foetal haemoglobin (HbF) on modifying SS has been increasingly recognised but it is clear that HbF levels are not the sole determinants of disease severity. For example adhesion of blood cells to endothelium also appears to be important. The factors examined in this thesis include the role of HbF in red cells, surface adhesion molecule (AM) expression on red cells and the effect of a number of endothelial factors.

Key goals in this thesis were to examine how HbF in red cells and the presence of AMs on red cells could interact as determinants of disease severity. In order to examine this interaction, it was first necessary to develop a technique to measure co-expression of AMs, HbF and RNA in the red cells. The first part of the thesis was therefore concerned with development of suitable methodology. A method of staining for surface red cell adhesion molecules and for internal HbF and RNA contents was successfully developed which has not been described previously. The relative effects of HbF expression and AM expression on survival of red cells beyond the reticulocyte stage were then examined in SS patients in steady state, in crisis and on HU treatment. The shedding of AMs by reticulocyte in culture was also examined in order to gain insight into the relative expression of AMs in mature red cell and reticulocyte populations.

6.2. KEY METHODOLOGICAL DEVELOPMENTS

Single-colour staining flow cytometry

The first new development in this thesis was to simplify the flow cytometric method for F^+cell assay obtained from Davis *et al*. 1998. The simplified procedure contains no washing step and gives comparable results with more convenience than previously. Either FITC or TC conjugated to monoclonal antibody against human HbF (MoAb-HbF-FITC or MoAb-HbF-TC) can be used without any difference in procedure nor results obtained. Three conventional (EDTA, citrate, heparin) and 1 special (CTAD) anticoagulants are all suitable for the procedure. The samples can be kept at 4°C for 2 weeks prior to test without any change in results obtained. Reproducibility indicated by intra- and inter-assay %CVs are acceptable (not exceed 10 and 20% respectively). The simplified procedure for F^+cells assay developed in this thesis is now available to be applied in any flow cytometric laboratory.

Double-colour staining flow cytometry

The second achievement in this thesis was the development of a double-colour staining flow cytometry for simultaneous assay of F^+cells, reticulocytes and F^+reticulocytes. The original procedure was taken from Dr. Bruch H. Davis (personal communication). However, the procedure was then simplified to contain no washing step and no volume calculation. This simplification needs less than one and a half hours to be ready for flow cytometry. All 4 anticoagulants mentioned before are suitable for the procedure. The reproducibility is acceptable, indicated by intra- and inter-assay %CVs less than 10 and 20% respectively. Samples can be kept at 4°C for 5 days prior to test. This is relatively convenient for the sample obtained on Friday afternoon to be kept in a refrigerator over the weekend to be performed on the next working day even on the longer holiday for up to 4 days. The results obtained from this procedure are relatively the same as the results obtained from single-colour staining. Furthermore, %reticulocytes obtained from this procedure are comparable to the results obtained from a reticulocyte analyser

(Sysmex SE-9500). This procedure is also ready to be installed in any flow cytometry laboratory.

Triple-colour staining flow cytometry

The third and probably the most important achievement in this thesis is the development of simultaneous triple-colour staining flow cytometry for measurement of the co-distribution of AMs (CD36, CD41 and CD49d). HbF (F^+cells) and RNA (reticulocytes). The 3 colours (green, red and deep-red) are used to stain RNA, AMs and HbF in the same assay. AMs, which are surface molecules, are stained first, follow by HbF and RNA, which are cytoplasmic molecules. Colour compensation has to be used in order to reduce the interference of one colour to another. This was done on a special program named "fast comp" in the computer, which controls all functions of flow cytometer. The results obtained from this procedure are consistent with the results obtained from the separated single-colour staining procedures. All 4 anticoagulants mentioned before are suitable for the procedure. The reproducibility is acceptable, indicated by intra- and inter-assay %CVs less than 10 and 20% respectively. Samples can be kept at 4°C for 3 days prior to test. This procedure can in principle be applied in any flow cytometry laboratory.

6.3. KEY FINDINGS

Contribution of foetal haemoglobin to sickle red cell survival

The findings in this thesis confirm that HbF and F^+cells are increased in SS patients and even more marked increase in HU-treated SS patients. Using the ratio of %F^+mature red cells to F^+reticulocytes recognised as the enrichment ratio (ER), it is clear that F^+cells survive longer than F^-cells, indicating an older age distribution of F^+cells than F^-cells. This is consistent with the finding that the patients who have higher %HbF or %F^+cells, normally have less clinical complications.

Contribution of adhesion molecules to survival of sickle red cells

The proportion of reticulocytes expressing AMs are much higher than the proportion of mature red cell expressing the same AMs. This finding is consistent with the knowledge that younger cells carry more AMs, since AMs are required for homing and maintaining cells within the bone marrow. Freshly released red cells (reticulocytes) still express surface AMs, and the *in vitro* culture of sickle reticulocyte shows that AMs are shed within 5 days of culture. However, this finding cannot rule out that AM expressing reticulocytes are preferentially sequestrated and removed from the circulation. Cells containing HbS polymers, become less flexible with slower transit through the microcirculation, and those which also express AMs, may be particularly likely to have shorter survival. Previous findings by other investigators that the proportions of reticulocytes and mature red cell expressing AMs in sickle cell patients are higher than in controls are consistent with the findings in this thesis.

In HU-treated patients, the proportion of mature red cell but not reticulocytes expressing AMs is reduced. This finding disagrees with the previous claim by Styles *et al.* 1997 that the reduction in AM expression is not caused by a decrease in reticulocytes in HU-treated patients. The findings in this thesis suggest that reduction in AM expression is secondary to the reduction of reticulocytes, since the % of reticulocytes expressing AMs is not significantly reduced after HU treatment. These findings are confirmed both in unpaired and paired studies.

Interactions of AMs and HbF on sickle red cell survival

In healthy control subjects, the proportion of F^+cells expressing AMs appears to be higher than for F^-cells. This applies for both reticulocytes and mature red cells. This finding suggests that F^+cells are possibly more primitive than F^-cells, because they carry more AMs, which could be an indirect indicator for a less differentiated population. This is also consistent with a lack of survival advantage for F^+cells over F^-cells in the control subjects. This is the first documented finding that F^+cells show any differentiating feature from F^-cells other than HbF content.

Unlike control subjects however, in sickle cell patients the proportions of cells expressing AMs is roughly equal in F+ve and F-ve populations. This indicates that HbF produces a benefit of prolonging red cell survival in SS patients. Because F^+cells have a survival advantage, and therefore an older age distribution, they have more time to shed AMs. Thus the higher AM expression which is seen in F^+cells of control subjects relative to F^-cells is counterbalanced in SS patients by the longer survival of F^+cells than F^-cells leading to a lowering of AM expression in F+ve relative to F-ve populations.

In HU-treated sickle cell patients, the proportion of F^+cells expressing AMs appears to be even lower than in SS patients without HU treatment. Examination of HbF content in red cells post-HU treatment shows more HbF per F^+cell, which would produce an additional survival benefit for such cells. Thus the effect of survival advantage leading to reduced AM expression is more pronounced than SS patients without HU treatment.

Endothelial, platelet and anaemic factors in sickle cell disorders

The original hypothesis which triggered the work in Chapter 5 was that as VEGF synthesis by endothelium is controlled by a hypoxia-sensitive element in vascular endothelial cells (VECs), microvascular occlusion and consequent tissue hypoxia in sickle cell disorders could lead to increased VEGF synthesis in VECs. Furthermore, as nitric oxide synthase (NOS) is also responsive to hypoxia, plasma levels of NOx (NO metabolites), might be predicted to be influenced by vaso-

occlusion. Thus in principle, plasma VEGF and NOx levels could be indicators of the degree of ongoing microvascular occlusion.

NOx levels in sickle cell patients and control subjects

In practice, NOx levels in plasma are likely to reflect a complex series of events. Factors affecting synthesis include local hypoxaemia in blood vessels, release of NO from platelets, endothelium and macrophages, hepatocytes and many other cells. NO synthesis is also affected by diet so that patients and control subjects having diversities in dietary intake could produce wide differences in plasma NOx levels. Blood NOx levels will also be affected by the rate of metabolism and interaction with other molecules such as haemoglobin. NO is known to be associated with Hb as nitrosyl-Hb and travel around the body within the red cells. NO may be stored intracellularly as nitrosothiol and dinitrosyl-iron-cysteine. Thus NOx levels in the plasma may not directly reflect the amount of NO produced at any moment.

The findings in this thesis of a lack of raised NOx in SS patients either in crisis or in steady state disagree with previous reports of either raised (Rees *et al.* 1995) or decreased (Lopez *et al.* 1996) levels of NOx. This is partly explained by the sample collection procedure. Serum and plasma NOx levels are unlikely to be secondary to platelet activation because control experiments in which platelets were activated *in vitro* did not increase NOx significantly. This suggests that other cells such as macrophages or circulating endothelial cells (Sowemimo-Coker *et al*, 1989) may be involved. However, this is speculative at present and the main conclusion of the work in this thesis is that serum and plasma NOx levels are not significantly modulated in SS patients.

VEGF levels in sickle cell patients and control subjects

Our initial finding that VEGF was raised in serum samples of SS patients could have been interpreted as consistent with the hypothesis that hypoxia upregulates VEGF production and release. However, it soon became apparent that *in vitro* activation of platelets was responsible for most of the measured serum and

plasma VEGF levels. This was not reported at the commencement of this work but has subsequently been confirmed by a variety of investigators in non-sickle patients. Interestingly, it appears that VEGF release *in vitro* is greater in SS patients than controls especially in serum samples, suggesting a greater tendency to platelet activation *in vitro* in sickle cell disorders. The reason for this has not been investigated in this thesis, but as platelet activation is a recognised feature of SS and sickle red cells or red cell spicules are known to interact with platelets. It is likely that this process occurs *in vitro* during collecting the serum samples.

When samples were collected into CTAD in order to inhibit platelet activation *in vitro*, VEGF remained high in SS patients compared with control subjects, although the difference was less marked. Causes of this raised VEGF in SS patients could in principle be due to generalised hypoxia, localised hypoxia with vaso-occlusion or to *in vivo* platelet activation. In order to address which of these was most responsible for raised VEGF levels, correlations were sought between VEGF and markers of hypoxia, *in vivo* platelet activation and endothelial activation. The results show no correlation of VEGF levels with plasma Epo, suggesting that generalised hypoxia secondary to anaemia was not responsible for the raised VEGF levels in SS patients. There was also no correlation of VEGF with markers of platelet activation (PF4 and BTG) or endothelial activation (sE-selecetin and sVCAM-1) despite there being good evidence for increased platelet and endothelial activation in SS patients compared to control subjects. The results in this thesis show that there are clearly increased VEGF levels in plasma, although the exact cellular origin remains obscure. It is also clear that there is an extensive endothelial and platelet activation in SS patients compared to control subjects. The lack of clear correlation with any particular factors suggests a complex interplay of both vascular endothelial cells and platelets.

6.4. FUTURE TRENDS

Application of flow cytometry in studies of sickle red cells

Phosphatidylserine (PS) exposure in sickle red cells has become an area of recent interest. PS exposure is known to activate coagulation in sickle cell patients (Wood *et al.*, 1996). Single-colour staining flow cytometry for PS exposure is now well established (Wood *et al.*, 1996). It would be valuable to know whether F^+cells show more or less PS exposure than F^-cells and whether this is associated with differences in activation of platelets and coagulation. The first question can in principle be answered by developing a double-colour staining flow cytometry for co-distribution of PS exposure (using annexin-V-conjugated FITC) and HbF content (using MoAb-HbF-TC). The preliminary result of this development is quite promising. However, the reproducibility of the procedure is yet to be improved.

Another area, which could be clarified using the flow cytometric staining developed in this thesis, is that of the relationship of HbF content to red cell density. Some ion-transporting channels, especially the Ca^+-dependent K^+ channel, which is responsible for ionic balance and cell density, do not function properly in sickle red cells (Franco *et al*, 1997). This malfunction leads to failure to maintain intracellular ionic balance that makes sickle red cells more dense. Some sickle red cells are dense at the time of release from the bone marrow (at reticulocyte stage) but some become more dense later (at mature red cell stage). The method developed in this thesis could allow examination of HbF content, RNA content and AM expression in subpopulations of cells previously separated by using centrifugation with density gradient media. This could show the effect of HbF content on red cell density both in reticulocyte and beyond reticulocyte stages. It would also be of interest to examine whether AM expression was similarly distributed between dense and less dense cells.

Markers of endothelial and platelet activation in sickle cells disorders

The findings in this thesis show clear evidences of endothelial and platelet activation in SS patients. These may be secondary to local vaso-occlusion but could also reflect a more generalised process. Any treatment that reduced vaso-occlusion, such as HU treatment, blood transfusion or bone marrow transplantation could reduce these effects and it would be of interest to study these systematically. A relatively less known area is the effect of anti-platelet drugs on severity of SS. Although there have been clinical studies undertaken on the role of aspirin in preventing vaso-occlusive crises (Greenberg *et al.*, 1983), there has been little systematic study of the effects of drug intervention on platelet and endothelial activation markers in SS patients. In addition to studying the effects of anti-platelet drugs on markers of endothelial and platelet activation, it would be of interest to compare these markers before and after HU therapy. The extent to which the rates of change in these markers with HU therapy parallel changes in AM expression and increments in F^+cells, could help elucidate the extent to which the benefits of HU result from changes in HbF or from changes in AM expression. Such studies would bring together the themes explored in chapter 4 and chapter 5.

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